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14. ABSTRACT
In this report, we have described the experiments pertaining to task#1 of our original proposal (To elucidate the significance of fetuin-A in breast carcinogenesis). The central hypothesis of this grant is that fetuin-A is a major serum derived growth factor for breast carcinoma cells and creates a favorable environment for the metastatic spread of tumor cells to the lungs. We have successfully placed $Fet^{+/+}/PymT^{+}$; $Fet^{+/-}/PymT^{+}$; and $Fet^{-/-}/PymT^{+}$ female C57 black mice in experimental protocols. Our data show that *lack of fetuin-A protects female fetuin-A null $PymT^{+}$ mice from breast cancer*. However, if fetuin-A null mice develop breast cancer, it is mainly squamous carcinoma, a rare breast cancer. We have also made significant progress in task # 2 where we now show for the first time that cell attachment is mediated only by fetuin-A and not its major contaminant which is alpha-2-macroglobulin. We also show both fetuin-A and alpha-2-macroglobulin can activate PI3 kinase/Akt.

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Role of Fetuin-A in tumor Cell growth (Progress Report) W81XWH-07-1-0254

Josiah Ochieng, Ph.D. (Principal Investigator)

Introduction:

In the last progress report (2008), there was no mention of $Fet^{-/-}/PymT^{+}$ (fetuin-A null) mice. At that time, we had obtained only the $Fet^{-/+}/PymT^{+}$ mice. In our experimental protocol, we needed at least 5 $Fet^{-/-}/PymT^{+}$ animals in order to obtain meaningful statistical data. I am happy to report that in this mid-term report we now have enough of the fetuin-A null, $PymT^{+}$ animals for proper analysis. The data we have obtained so far clearly show that fetuin-A plays a significant role in breast carcinogenesis. The $PymT^{+}$ mice that are also fetuin-A null rarely develop breast cancer and when they do, the tumor development is significantly delayed, suggesting that the lack of fetuin-A lengthens tumor latency in these animals. More importantly the type of breast cancer that develops in fetuin-A null mice in most cases is unique in that it tends to be squamous cell carcinoma with a large number of keratin pearls. We are in the process of doing more experiments to determine why this happens. We are also asking whether this is a survival strategy for this type of breast cancer.

In our working hypothesis we had envisioned that fetuin-A is a major driver of PI3 kinase/Akt signaling during breast carcinogenesis [1]. However, in present studies, activated Akt although slightly higher in $Fet^{+/+}$ and $Fet^{-/+}$ animals, is also present in $Fet^{-/-}/PymT^{+}$ (null)mice. In an attempt to search for a more plausible mechanism to explain the present data, we questioned the interplay between fetuin-A and TGF-beta signaling. The current dogma states that in breast cancer, TGF-beta plays a dual role; it suppresses oncogenic transformation but also promotes the progression and metastasis once transformation has occurred [2]. Fetuin-A has been shown to be TGF-beta receptor mimic that can mop TGF-beta away from the breast epithelial cells in both the wild-type and heterozygous mice. We therefore postulate in the present work, that TGF-beta signaling is down regulated during the early stages of tumor initiation (30-90 days old) in both $Fet^{+/+}/PymT^{+}$ and $Fet^{-/+}/PymT^{+}$ mice by fetuin-A. This in turn removes the breaks that slow down transformation. However once the tumors develop and grow larger, the levels of fetuin-A in the serum is significantly decreased and TGF-beta signaling is turned on in these cells. At this point TGF-beta acts as a tumor promoter [2]. Apart from mopping the TGF-beta to allow unhindered transformation and promotion of breast cancer, fetuin-A may also be involved in the progression of breast tumors by promoting anchorage independent growth of tumor cells as explained below. In $Fet^{-/-}/PymT^{+}$ mice on the other hand, lack of fetuin-A means that TGF-beta is readily available to the tumor cells and suppresses transformation and promotion [2], but unfortunately if the tumor develops, it quickly adopts a more aggressive phenotype that does not need fetuin- A or any other pathway supported by fetuin-A for growth.

We have also made significant progress in specific aim # 2 that constituted task # 2 in our statement of work. Basically we can report that serum from both $Fet^{+/+}/PymT^{+}$ and $Fet^{-/-}/PymT^{+}$ mice support the growth of breast carcinoma cells on plastic. However, other studies initiated in our laboratory point to serum exosomes as the likely growth platforms that affect anchorage independent growth of breast tumor cells *in vivo*. We have shown that these exosomes are associated with fetuin-A.

BODY:

Task # 1 (To elucidate the significance of fetuin-A (ahsg) in breast carcinogenesis). In this task, our goal was to cross $Fet^{+/+}/PymT^{+}$ mice with $Fet^{-/-}/PymT^{+}$ resulting in progeny

that are $Fet^{+/+}/PymT^{+}$; $Fet^{-/+}/PymT^{+}$; and $Fet^{-/-}/PymT^{+}$ mice. One problem that plagued us throughout this project was the slow pace at which we obtained littermates that were $Fet^{-/-}/PymT^{+}$ from the breeding protocols to be moved to the experimental groups. Despite these difficulties, I am happy to report that in the past one year, we have obtained over 10 $Fet^{-/-}/PymT^{+}$ mice that have been successfully moved to the experimental protocols.

One clear outcome from this study that we can now report is that female mice that are $PymT^{+}$ but lack fetuin-A are highly protected from breast cancer. The data we have obtained thus far support our hypothesis that fetuin-A is a major player in breast tumorigenicity. We are presently preparing the report to be submitted to Cancer Research. In the following report, we have followed 21 mice divided into the following experimental groups:

Genotype	Number of mice
$Fet^{+/+}/PymT^{+}$	7
$Fet^{+/-}/PymT^{+}$	7
$Fet^{-/-}/PymT^{+}$	7

Lack of fetuin-A prolongs tumor latency in animals: We have maintained this survival curve for the past 8 months as we follow the mice in the above table. The ordinate stands for the percentage of mice that are tumor free at a given age group (abscissa). The mice that had palpable breast tumors were allowed to live until their tumor reached a size of 15 mm or showed signs of discomfort as stipulated in our animal experimental protocols. At this point the mice were sacrificed by CO_2 asphyxiation and the tumors removed for histopathology.

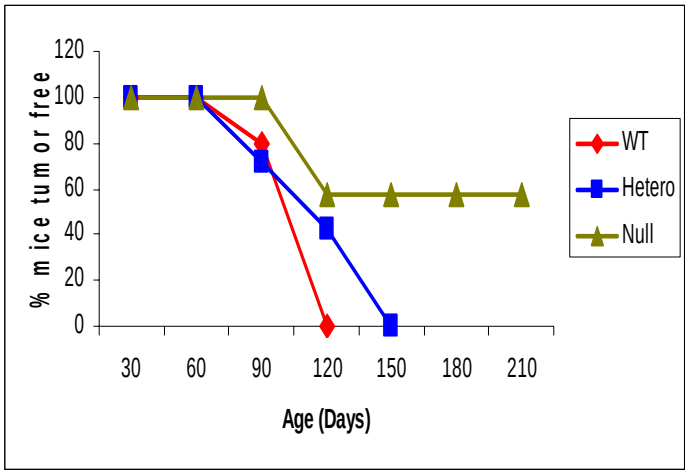


Fig. 1. Survival curve of female $PymT^{+}$ in experimental groups. Tumor was recorded as pulpable mass in the breast area. By 90-days of age, a number of wild-type and heterozygous mice had pulpable tumor and the mice were sacrificed once the tumor reached 15 mm in length. By 180 days, only the fetuin-A null mice were alive because they were tumor free.

Both the wild-type ($Fet^{+/+}/PymT^{+}$) and Hetero ($Fet^{+/-}/PymT^{+}$) rarely live past 150 days without showing evidence of heavy tumor growth. However, even as this report is being written, we have 4 fetuin-A null ($Fet^{-/-}/PymT^{+}$) that are over 180 days old and tumor free. Furthermore we sacrificed fetuin-A wild-type, hetero and null $PymT^{+}$ mice at 30, 60 and 90 days of age and carefully performed histopathological evaluation of their breast tissues. In $Fet^{-/-}/PymT^{+}$ animals at 60 days, when the H&E tissue sections were examined, they consisted predominantly of adipose tissue interspaced with a few acini, ducts and blood vessels that were randomly scattered. The $Fet^{+/-}/PymT^{+}$ mammary tissues on the other hand at 60 days show an expansile mass with pushing margins and consists of neoplastic cells arranged as solid sheets of epithelial cells, multifocal areas of glandular differentiation and papillary carcinoma separated by various quantities of fibrovascular stroma and adipose tissue. Similarly, majority of 60 days old mammary tissue of $Fet^{+/+}/PymT^{+}$ show multifocal areas of

hyperplasia characterized by acini proliferation and focal areas of typical Min characterized as early expansile carcinoma with atypical pleomorphic nuclei along one of the margins of the lesion (data not shown). At 90 days of age, the mammary tissues of the fetuin-A null animals still show predominantly adipose tissue interspaced with a few acini and ducts (Fig.2). Majority of $Fet^{-/+}$ mammary tissues at 90 days of age consist of expansile masses characterized by extensive areas of necrosis and scattered islands of neoplastic cells with a central fibrovascular core arranged in a medullary pattern. Same pattern is seen in $Fet^{+/+}$ mammary tissues at 90 days (Fig. 2).

Fetuin-A null mice that formed tumors, did so at around 120 days. A typical mammary section at this age consist of expansile mass characterized by extensive areas of necrosis and scattered islands of neoplastic cells with a central fibrovascular core arranged in a medullary pattern. Others consist of cystic acini that are filled with pink proteinous material and lined by neoplastic cells exhibiting cellular atypia and papillary projections into the lumen. The neoplastic cell layer adjacent to the necrotic debris show squamous metaplasia with parakeratosis with retention of keratinocyte nuclei. Keratin pearls characterized by small concentric lamellae of keratin aberrantly produced within the tumor are evident and are present in the necrotic debris (Figs. 3 and 4). A typical $Fet^{-/+}/PymT^{+}$ mammary tissue at 120 days of age consist of multifocal areas of glandular differentiation and papillary carcinoma separated by various quantities of fibrovascular stroma and adipose tissue. Neoplastic cells are arranged as solid sheets and in some areas exhibit intraluminal epithelial proliferation characteristic of MIN. The neoplastic cell layer adjacent to the necrotic debris show squamous metaplasia with parakeratosis with retention of keratinocyte nuclei (Fig. 3).

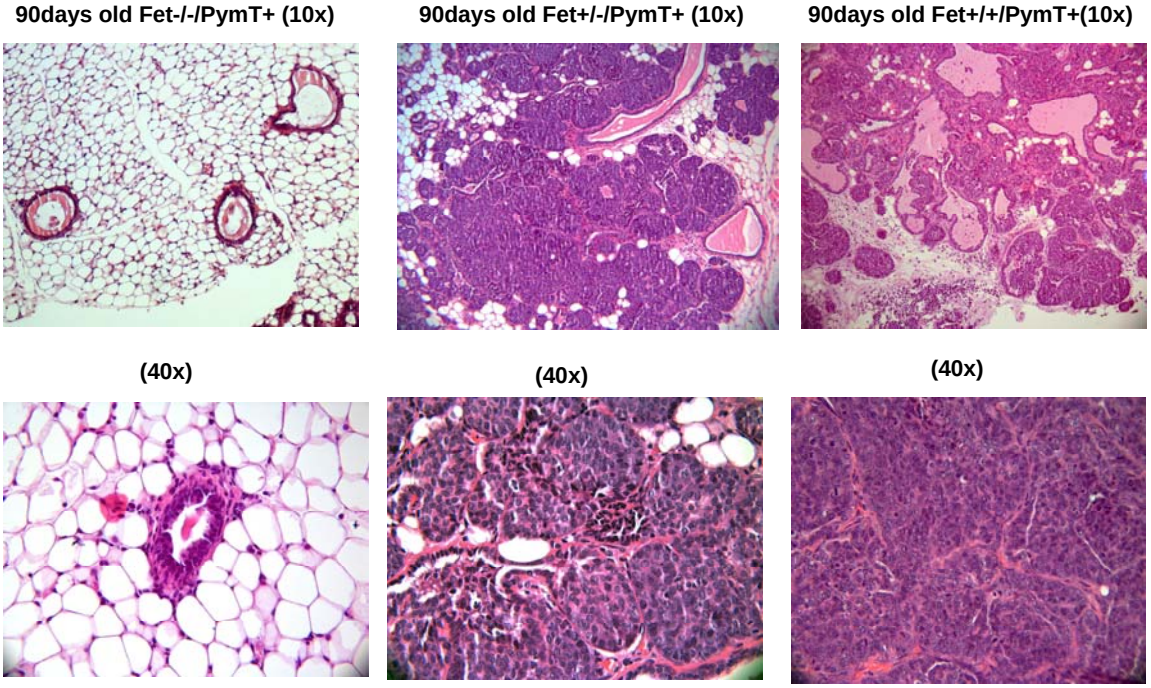


Fig 2. Histo-pathological staining of breast tumor (H&E) obtained from 90 days old mice with undetectable tumors (pulpable). In the top panels, both the heteros and wild type mice had extensive mammary intraepithelial neoplasia (MIN) or ductal in-situ carcinoma (DIC) that could also be felt as nodules under the skin. The null mice on the other hand, had normal ducts at this age that was not pulpable.

A typical mammary tissue of $Fet^{+/+}/PymT^{+}$ mice at 120 days consists of expansile mass with pushing margins and consists of neoplastic cells arranged as solid sheets of epithelial cells, multifocal areas of glandular differentiation and papillary carcinoma separated by various quantities of fibrovascular stroma and adipose tissue. Neoplastic cells have infiltrated surrounding adipose tissue frequently showing acini formation (Figs 3 and 4)

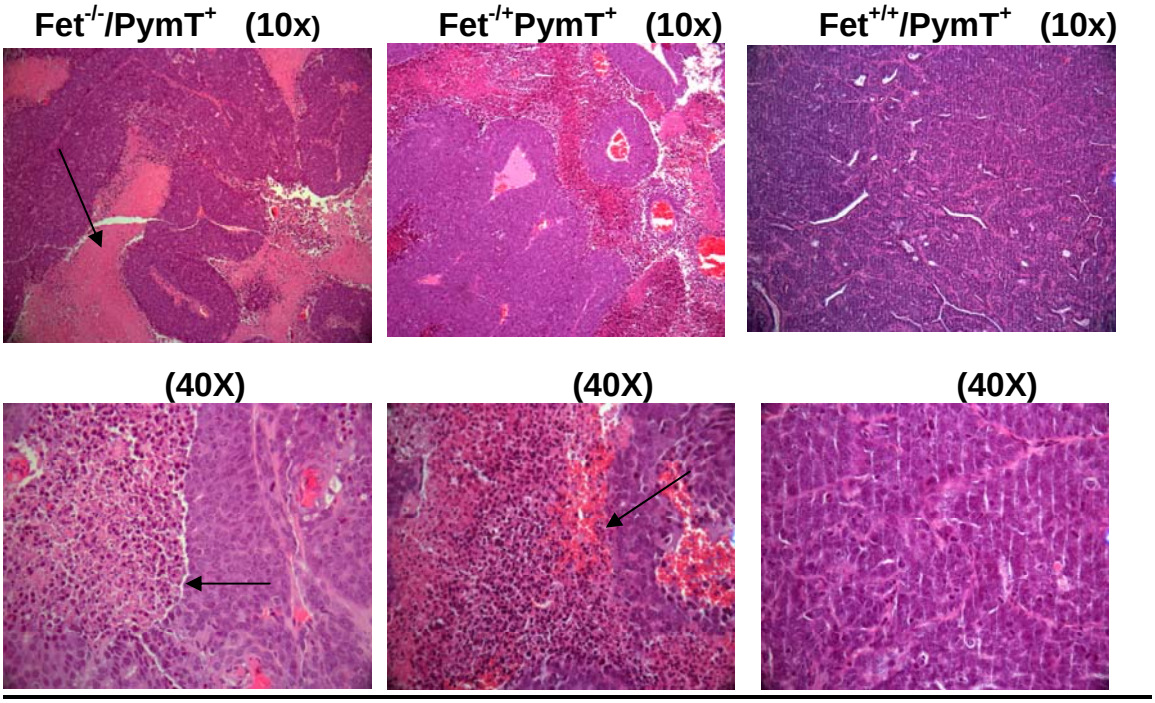


Fig. 3. The H&E staining of breast tumor tissues from fetuin-A null ($Fet^{-/-}/PymT^{+}$), heterozygous ($Fet^{+/-}/PymT^{+}$) and wild-type ($Fet^{+/+}/PymT^{+}$) mice. The arrows show areas of wide-spread necrosis mostly in the fetuin-null tumors and to a lesser extent in hetero tumors. The wild-type tumors are mostly solid adenocarcinoma as shown.

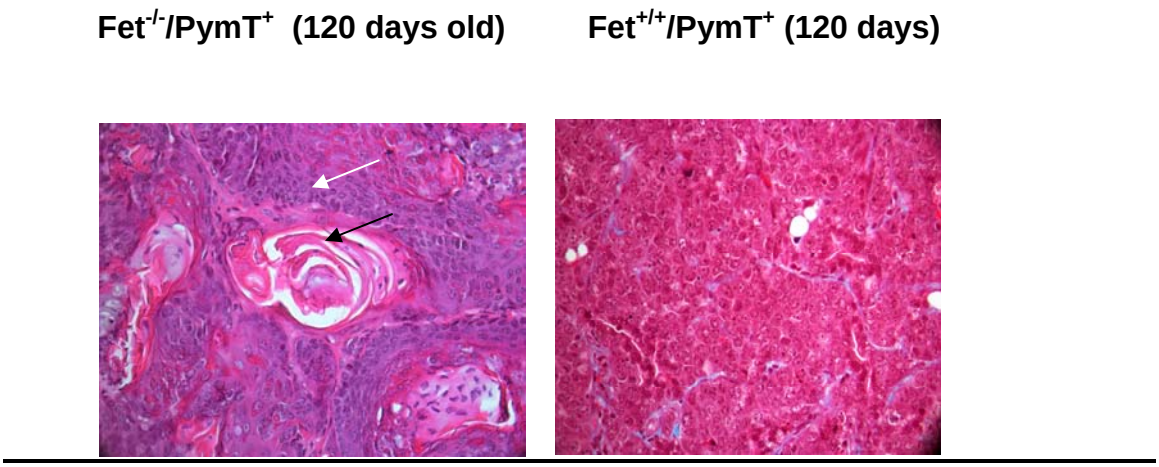


Fig. 4. Histopathology of 120 days old breast tumors in both fetuin-A null and wild-type mice. The tissues sections were stained with trichrome. This stains collagen fibers a bluish color and keratin red. The panel on the left (fetuin-A null) shows keratin pearls, a feature rarely seen in breast tumors. All

the fetuin-A null tumors we have obtained thus far show keratin pearls and extensive necrosis. This is not seen in the wild-type tumors (right panel)

PI3 Kinase/Akt signaling in fetuin-A wild-type vs fetuin-A null mammary tissues

We hypothesized that since fetuin-A activates the PI3 kinase/Akt signaling pathway, there would be little or negligible phospho-Akt (S473) in either normal or tumorigenic mammary tissues of fetuin-A null mice. On the other hand, there should be heavy staining of phospho-Akt (S473) in either normal or tumorigenic mammary tissues of fetuin-A wild-type mice. The data we obtained, however, was inconclusive. We stained many sections and determined that in most normal tissues, phospho-Akt (S473) staining was weak (low activity of the pathway) in the normal mammary tissues of the fetuin-A wild-type, hetero and null animals (Fig. 3, upper panels). However, in tumorigenic mammary tissues, there was heavy staining of phospho-Akt (S473) in all the animals (Fig. 5, lower panels). Even though the mammary tissues of $Fet^{+/+}$ and $Fet^{-/+}$ PymT animals appeared to have a heavier staining of phospho-Akt (S-473) compared to the $Fet^{-/-}$ animals, there was no consistency because for some $Fet^{-/-}$ mammary tissues the intensity of phospho-Akt staining was basically similar to those of $Fet^{+/+}$ and $Fet^{+/-}$ animals. For this reason, it is not appropriate to claim that the decrease in tumor latency seen in fetuin-A wild-type and heterozygous animals was due to the activation of PI3/Akt signaling alone. Therefore, as an alternative strategy, we examined the TGF-beta signaling in the $Fet^{+/+}$; $Fet^{+/-}$ and $Fet^{-/-}$ $PymT^{+}$ mice at different stages of mammary tumorigenicity. The rationale for this decision was based on published information showing that fetuin-A has a high capacity to influence TGF-beta signaling in normal and cancerous mammary tissues in vivo [2].

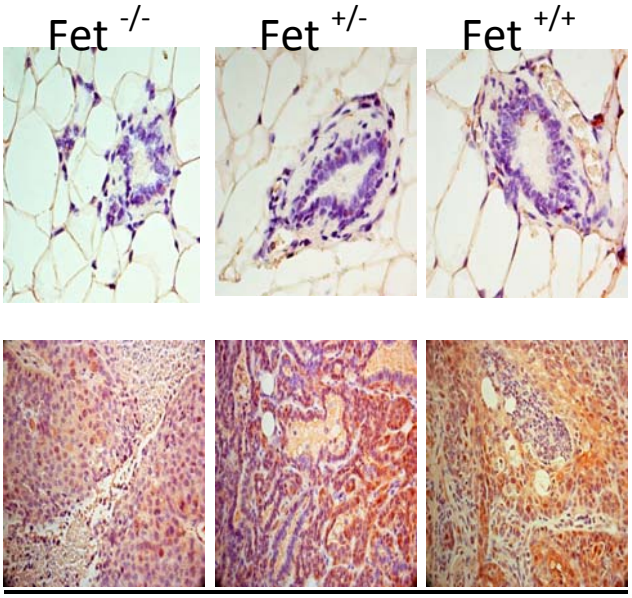


Fig. 5. Expression of phospho-Akt (Ser-473) in mammary tissues of $Fet^{-/-}$; $Fet^{+/-}$ and $Fet^{+/+}$ $PymT^{+}$ mice. Mammary tissues were processed, embedded in paraffin and sectioned as described above. The sections were de-waxed, and processed for immunohistochemistry. The upper panels represent mammary tissues from the animals at early time points (30 day old mice) while the lower panels represented tumors obtained from 150 days old mice. The slides were incubated with antibodies to phospho-Akt (Ser-473) followed by secondary antibodies and then the substrate for horse-radish peroxidase added.

TGF-beta signaling and breast cancer: We have demonstrated that fetuin-A mediates the PI3 kinase/Akt signaling in human mammary tumor cells in vivo [1], and therefore wondered whether the same mechanisms were at play during mammary carcinogenesis *in vivo*. We

thus predicted that the fetuin-A wild-type and to a lesser extent hetero mice that were also $PymT^{+}$ would have enhanced PI3 kinase/Akt signaling relative to the null mice. The tissues of 60 and 90 days old mice were stained with antibodies to activated Akt (Akt-tyrP473). The data showed that whereas there was a slight increase in PI3 kinase/Akt signaling in mammary tumor of both the $Fet^{+/+}$ and $Fet^{-/+}$ PymT mice (Fig 5), this alone could not account for the dramatic increase in the latency of mammary tumors in fetuin-A null mice.

A number of studies have demonstrated that in breast cancer, during the early stages of transformation, TGF-beta signaling slows down transformation thereby acting as a tumor suppressor, for review see [2]. TGF-beta is secreted by both the stromal fibroblasts (which cross talk with epithelial cells) and the breast epithelial cells proper. In the extracellular milieu TGF-beta binds to a number of proteins including fetuin-A that acts as TGF-beta receptor mimic [3]. It is therefore conceivable that during early stages of breast carcinogenesis, the presence of fetuin-A may in fact divert TGF-beta away from the breast carcinoma cells which would allow the unabated transformation of the breast epithelial cells. The absence of fetuin-A in the null mice on the other hand would ensure adequate bioavailability of TGF-beta to the breast epithelial cells thereby putting a break on their transformation potential. Based on this information, we examined the expression of phosphorylated smad2/3 in the breast tissues of $Fet^{-/-}/PymT^{+}$, and $Fet^{+/+}/PymT^{+}$ as a readout of TGF-beta signaling. The data indeed suggest that this is the mechanism that is likely to be responsible for delayed transformation in fetuin-A null mice (Fig. 6)

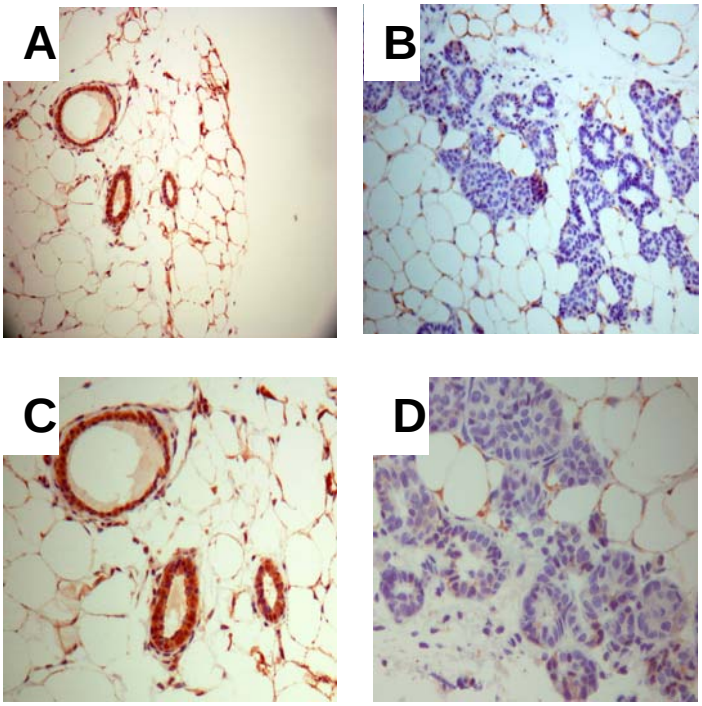


Fig. 6. Phospho-smad2/3 expression in breast tissues of fetuin-A null and wild-type $PymT^{+}$ mice. Female littermate mice were sacrificed at 60-days of age and breast tissue processed for immuno-histochemistry. The tissues were incubated with antibodies to phosphosmad2/3, followed by secondary antibodies. Of note is the intense staining of phospho-smad2/3 in the ducts of fetuin-A null mice (Panels A and C). Negligible staining was observed in the ducts of fetuin-A wild-type mice which showed a high degree of hyperplasia at this early stage (Panels B and D). Upper panels (10X) and lower panels (20X)

All the four $Fet^{-/-}/PymT^{+}$ mice that developed palpable tumors were at least 120 days old. When the tumors develop in these mice, they tend to be more aggressive compared to the wild-type tumors based on pathological parameters. It also appears that when these tumors develop, the TGF-beta may in fact fuel the progression of the tumors. A similar mechanism could also enhance the progression of the wild-type ($Fet^{+/+}/PymT^{+}$) tumors. It has been documented that fetuin-A levels decrease as tumors progress [4]. This could explain the intense phospho-smad signaling in some areas of the wild-type tumors (Fig. 7). It is evident

that once tumors form in the $Fet^{-/-}/PymT^{+}$ mice, they proliferate as rapidly as the wild-type or the heteros. It appears that these tumors adopt a novel pathway of progression which could shed new light into etiology of the different types of breast cancers including the so called triple negatives [5]. We are now in the process of isolating mRNA from both the wild-type and fetuin-A null tumors for DNA micro-arrays studies to ascertain the pathways that are up-regulated or down-regulated in fetuin-A null tumors relative to the fetuin-A wild-type breast tumors.

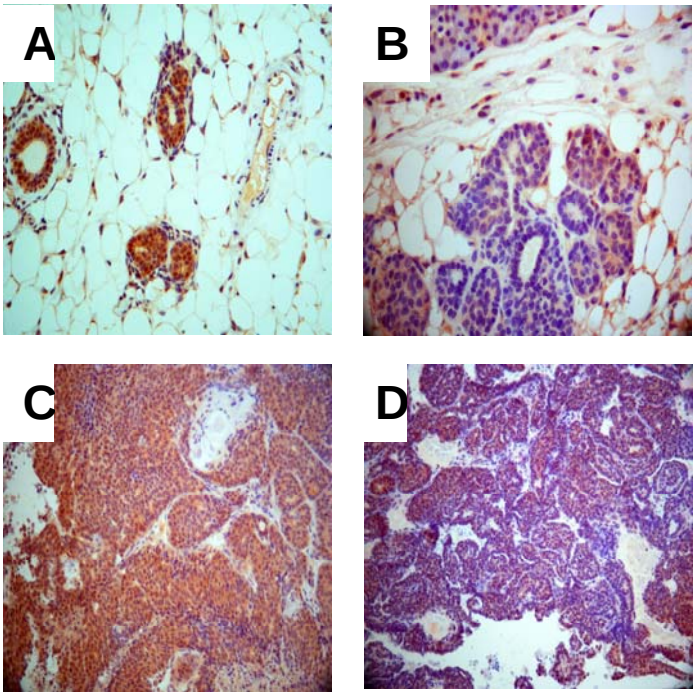


Fig.7. Phospho-smad2/3 expression in breast tissues of fetuin-A null and wild-type $PymT^{+}$ mice. Female littermate mice were sacrificed at 90 (Panels A and B) and at 120 (Panels C and D) days of age and breast tissue processed for immuno-histochemistry. The tissues were incubated with antibodies to phosphor-smad2/3, followed by secondary antibodies. Of note is the intense staining of phosphor-smad2/3 in the ducts and tumor tissue of fetuin-A null mice (Panels A and C, respectively). Some staining was evident in tumor tissues of fetuin-A wild-type mice (Panels B and D)

In conclusion, we have gained new information from the histopathology and immunohistochemistry of the mice tumors with and without fetuin-A. In all the cases examined, the tumors from fetuin-A null ($Fet^{-/-}/PymT^{+}$) null mice demonstrated a high degree of necrosis (Fig. 3, arrow). The explanation for this is not conclusive but we hypothesized that due to their aggressive growth, the tumor cells quickly overwhelmed the blood supply, resulting in necrosis. Also observed mostly in the fetuin-A null and hetero tumors, was a high degree of infiltration by leukocytes which presumably go in to clean up the necrotic tumor cells (Fig.3, arrows in lower panels). Nevertheless this work is on going and we hope to identify the specific cell types that are involved.

Progress on Task # 2 (***To define the role of fetuin-A in the in vitro growth and signaling of human breast carcinoma cells (months* 18-36**)). In this aim we hoped to understand the signaling mechanisms by which fetuin-A regulates tumorigenicity both *in vitro* and *in vivo*. The problem that has plagued fetuin-A research for a number of years is the inability to isolate this protein in a pure form. The fetuin-A obtained by ammonium sulfate precipitation of serum proteins (Pedersen method) is always contaminated with proteins such as alpha-2-macroglobulin. In our protocol we suggested that we would use WGA-column. Even though

this is a popular method of fetuin-A purification, contaminants in the purified fractions are still detectable in coomassie (Fig.8). The position of fetuin-A on the gel is shown by the arrow.

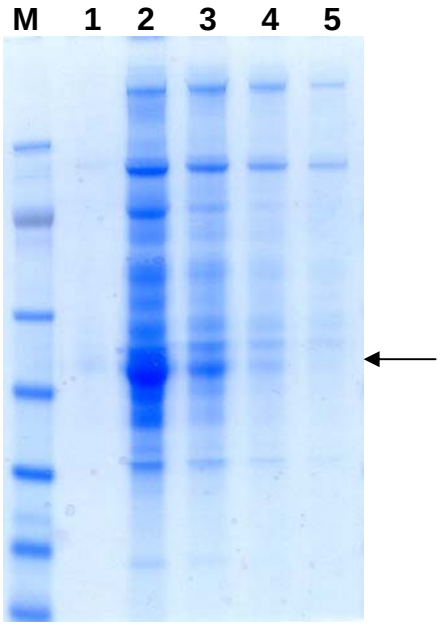
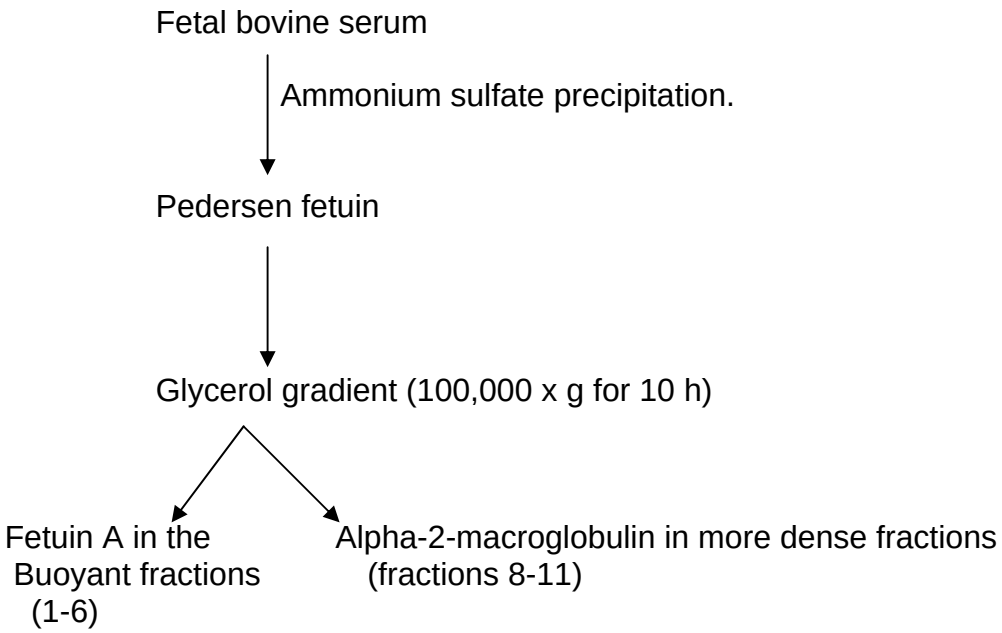


Fig. 8. Purification of Pedersen fetuin-A on WGA-affinity Chromatography. Pedersen fetuin (ammonium sulfate precipitation of fetal bovine serum) was incubated overnight with WGA-agarose, washed with PBS, packed in a column ( ) and then equilibrated by the addition of two more column volumes of PBS. The fetuin-A was eluted with 0.2 M of N-acetylglucosamine in PBS and I ml fractions collected. As shown most of the protein eluted in fraction 2. The fetuin-A band is observed at ~ 50 kDa. As can be seen, this WGA column is incapable of separating the major contaminants of fetuin-A. The upper bands represent alpha-2-macroglobin. The major band eluted in tube # 2 is relatively impure. The position of fetuin-A is shown by the arrow (~50 kDa).

Thus with this level of impurity, it is difficult to ascertain whether any growth related signaling is directly attributable to fetuin-A. We hypothesized that fetuin-A is a major activator of the PI3 kinase/Akt signaling pathway. However, since we show that fetuin-A purified by majority of the methods including Pedersen, co-purify with alpha-2-macroglobulin (another activator of the PI3 kinase/Akt pathway), we had to devise another purification method. We spent almost 7 months investigating other methods. We finally settled on glycerol gradient centrifugation which was the only method thus far capable of separating alpha-2-macroglobulin from fetuin-A (Fig. 9). The following flow chart describes the procedure



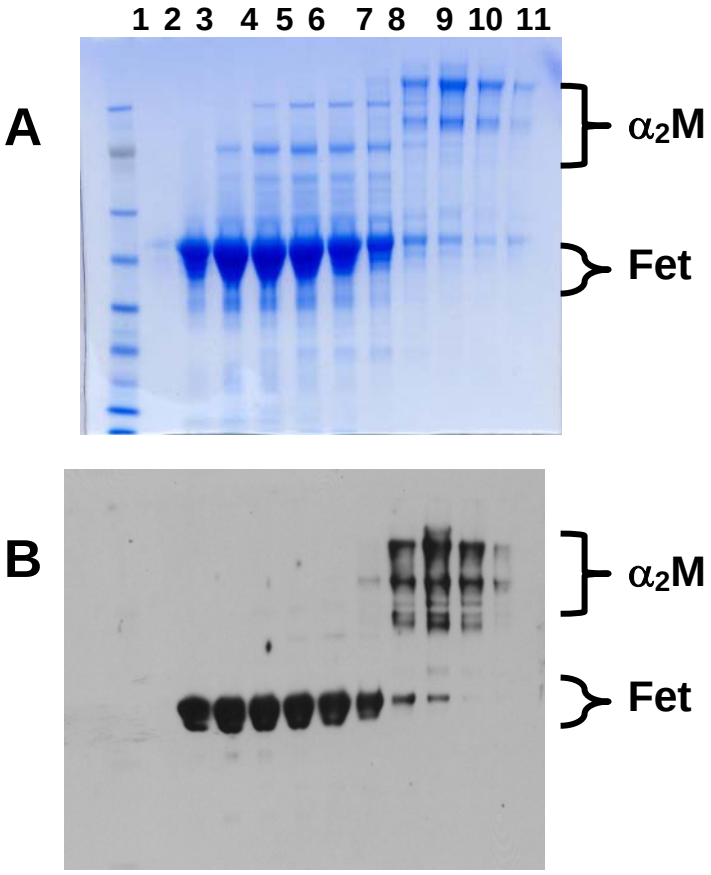


Fig. 9. Analysis of Pedersen fetuin-A by glycerol gradient centrifugation.

Pedersen fetuin (100 mg) was dissolved in a minimal volume of buffer and then layered on a gradient of glycerol (10-60%). This was then subjected to ultracentrifugation (100,000 x g) for 10 hours. Equal volumes of glycerol gradient fractions were analyzed by SDS-PAGE in 4-12% gradient gels and either stained with colloidal Coomassie (panel A) or blotted unto nitrocellulose membranes and probed with antibodies to alpha-2-macroglobulin and fetuin-A.

Of note is the alpha-2-macroglobulin peak in fractions 9 (S3) and the fetuin peak at fractions 4-5. Fraction # 2 had a single band in both Coomassie and western. This is the first time we have been able to achieve this level of purification.

With the glycerol gradient approach we have been able to purify fetuin-A to yield a single band (lanes 1 and 2) in both the coomassie and western blot using fetuin-A antibody which usually picks other bands due to contaminants like alpha-2-macroglobulin. Glycerol was removed from the fractions by dialysis followed by concentrations such that each tube was 1 mg/ml of protein. The fetuin-A purification studies, address **Task 2a** (modified **SOW**). We then questioned which fraction had the ability to support the attachment/adhesion of breast cancer cell lines. Each fraction was tested individually (Fig. 10). As shown, the fractions with pure fetuin-A (one band in coomassie) supported the most attachment. There was virtually no attachment when the alpha 2 macroglobulin enriched fractions were incubated with the cells in the presence of Ca^{2+}. We also pooled the fractions and renamed them S1 (lanes 1-3); S2 (lanes 4-7) and S3 (lanes 8-11). These were also tested for their abilities to support attachment and again the fetuin-A enriched pools supported attachment while alpha-2-macroglobin enriched pool (S3) did not (Fig. 10, panels B and D). These studies address **Task 2b** (modified **SOW**).

Using the same procedures, we will purify human fetuin-A from human serum. The serum will be purchased from Sigma and then subjected to ammonium sulfate precipitation to get the crude protein also known as the Pedersen fetuin. This product will be dialyzed, dissolved in minimal amount of HBSS and then subjected to glycerol gradient centrifugation as described above. This will be **Task 2C** (modified **SOW**)

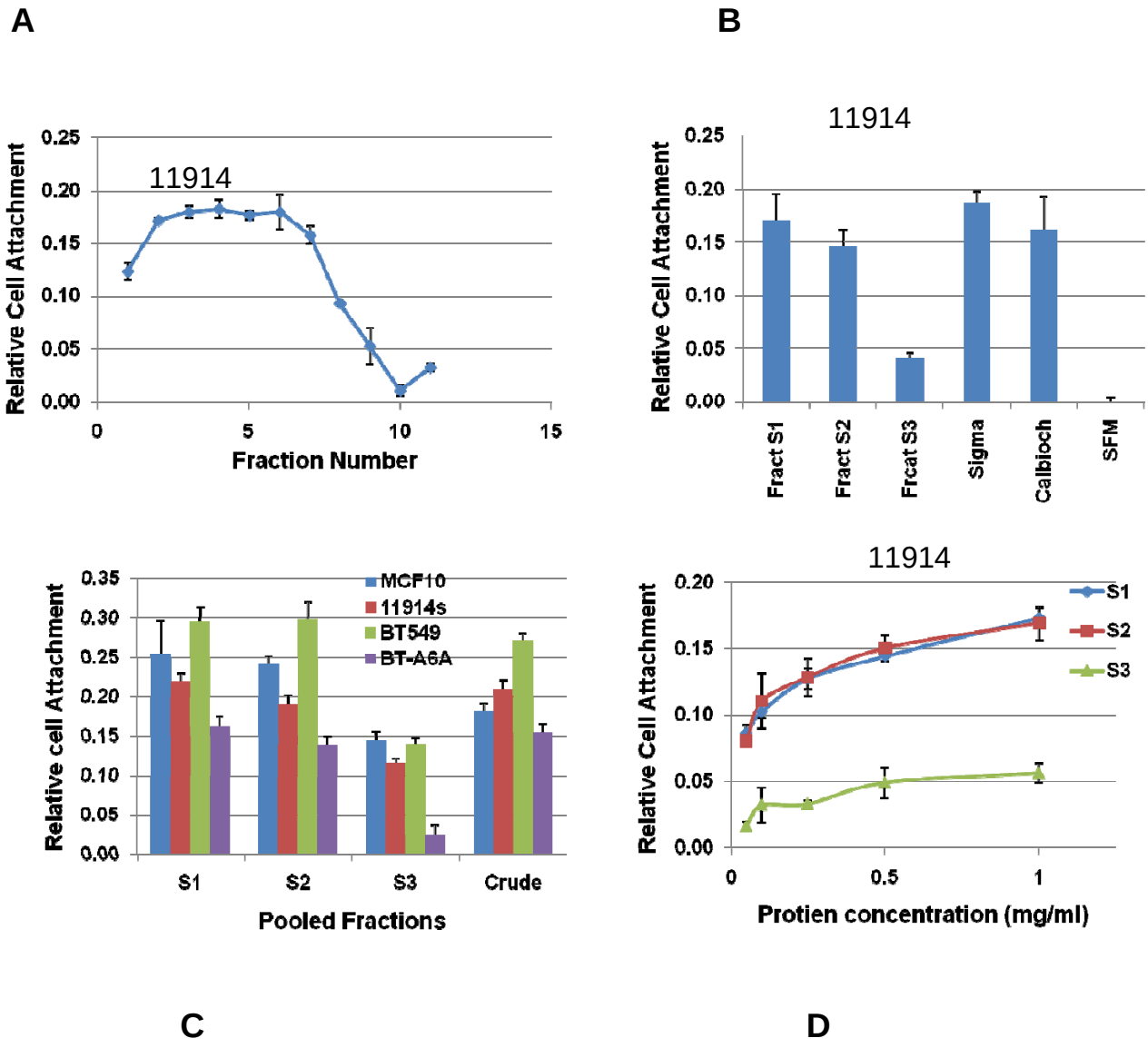


Fig. 10. Cell attachment assays using glycerol gradient purified fetuin-A. Perdesen Fetuin-A from bovine serum (sigma) at 50 mg/ml in TBS was layered on a 10-60% glycerol step gradient, centrifuged and equal fractions collected from the top. Triplicate wells of a micro-titer plate were coated overnight at 4^{o}C with 100 µl of a 1:2 dilution of each fraction (Panel A) or pooled and dialyzed fractions S1, S2 and S3 (0.5 mg/ml) (panels B-D). BT549 cells stably expressing Galectin-3 (clone 11-9-1-4) (panels A-C or the indicated breast cancer cell lines (panel C) were harvested, washed and re-suspended in serum free medium. About 10e4 cells were then plated on the pre-coated wells and these allowed to attach overnight at 37^{o}C in the presence of Ca^{2+} ions. Unattached cells were

removed along with the medium by aspiration and cells counts estimated using the Alamar blue assay [1]. The ordinates represent attachment of cells relative to attachment in serum-free medium.

Having established that fetuin-A and not its major contaminant (alpha-2-macroglobin) is responsible for cellular attachment, we next questioned whether fetuin-A mediates the growth related signaling mechanisms such as MAP kinase/ERK or PI3 kinase/Akt. We clearly demonstrated that the purified fetuin-A activates the PI3 kinase/Akt, using P-Akt (S473) as the readout (Fig. 11) as previously reported by our laboratory [1]. Interestingly alpha-2-macroglobulin also activates PI3 kinase/Akt [6] and so if these two proteins are together the activation of PI3 kinase/Akt can by synergistic. One other interesting observation, was the ability of purified fetuin-A to inhibit the activation of MAPK in the breast carcinoma cells. It appears that fetuin-A temporarily binds (mops) most of the extracellular Ca2+ with a concomitant reduction in the activation of MAPK. This experiment will be repeated with longer time point to define this mechanism. These studies, using purified fetuin-A, address **Task 2b** (modified **SOW**).

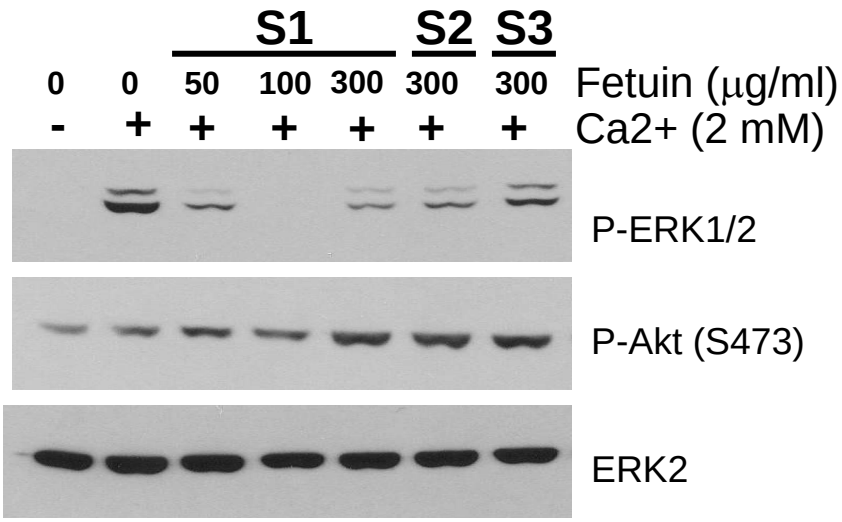


Fig. 11. Fetuin diminishes Ca^{2+} dependent activation of MAPK in breast carcinoma cells. BT549 cells were serum-starved for 24 h, then washed 2x with HBS and incubated in HBS containing 2 mM Ca^{2+} and the indicated concentrations of the pooled glycerol gradient fractions of fetuin-A for 10 min. Cells were harvested using a cell scrapper, washed with ice-cold HBS and lysed in ice-cold RIPA buffer containing protease and phosphatase inhibitors. Equal amounts of proteins were analyzed by western blotting using antibodies to phosph-ERK1/2, phospho-Akt (S473) and total ERK2.

Interestingly, when we purified exosomes (nanovesicles found in serum), they separated in the same fractions in which fetuin-A is found (Fig.12). Serum exosomes do indeed contain fetuin-A and we postulate that serum exosomes that promote the anchorage independent growth of breast tumor cells use fetuin-A to signal this growth promoting ability. Our laboratory, we believe, is the first to report this interesting observation [7].

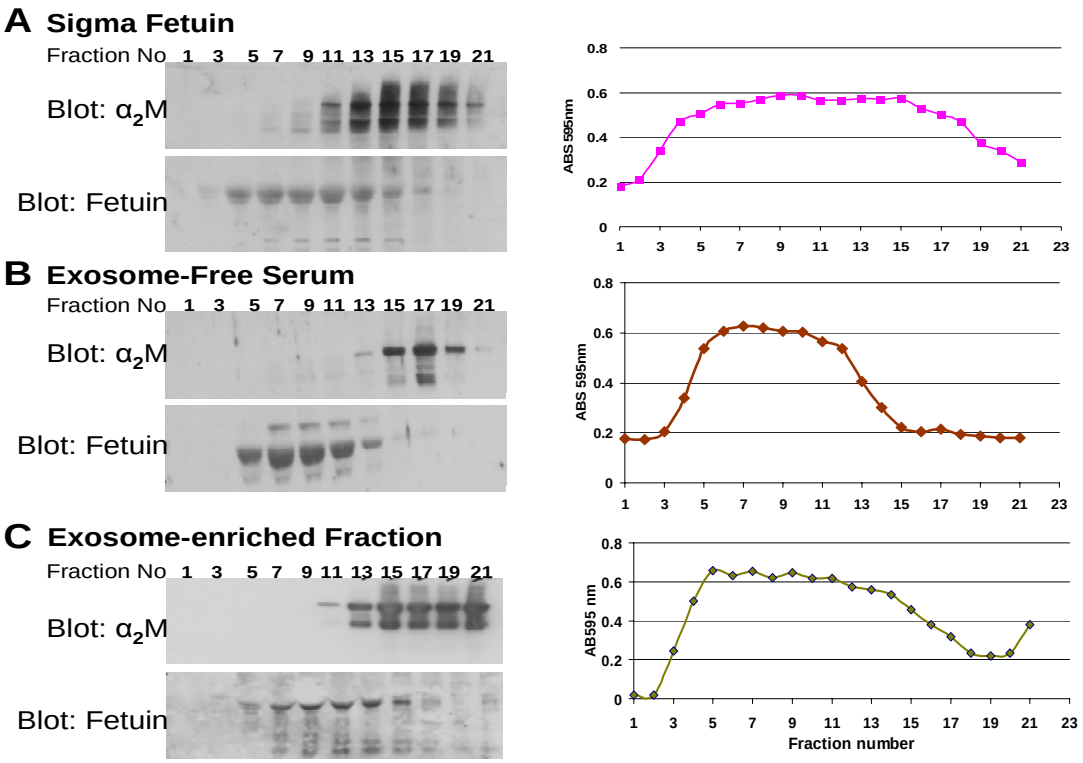


Fig. 12. Purification of fetuin-A and fetuin-A containing exosomes on glycerol gradients. The fetuin-A purchased from sigma (Pedersen fetuin-A) was layered on a glycerol gradient and centrifuged overnight at 100,000 x g. The fractions (every other fraction) obtained from top of the tube to the bottom were then assayed for alpha-2-macroglobulin (α_2M) and fetuin-A by western blot. As can be seen in all the three preparations, tubes 3-7 contained only fetuin-A, affording a very convenient way to separate the two proteins.

This work is currently ongoing and will be reported within the next reporting period. We hope to purify at least 100 mg of fetuin-A from both human and bovine sera and the purity will be ascertained by both colloidal-coomassie staining and western blotting techniques. We will then use these purified proteins to define fetuin-A signaling mechanisms.

Pitfalls: The major pitfall has continued to be the slow pace at which we obtain fetuin-A null mice that are also PymT$^+$. The research has opened up very interesting question and so we will continue to generate these mice to obtain serum for future studies. We have enough fetuin-A heterozygous and wild-type mice as well as their serum. The purification of fetuin-A has been a challenge but we finally found a viable alternative and so we hope to complete the experiments designed for Aim # 2 (Task # 2) in the next 10 months.

KEY RESEARCH ACCOMPLISHMENTS:

- Our studies for the first time demonstrate that fetuin-A/alpha 2HS glycoprotein as a promoter of breast carcinogenesis *in vivo*.

- We have demonstrated that fetuin-A mediate breast carcinogenesis by modulating TGF-beta and PI3 kinase/Akt signaling.

- We conclusively demonstrate that fetuin-A is the major cell adhesion protein in serum.

- We have shown for the first time that serum exosomes that are also rich in fetuin-A are needed to maintain anchorage independent growth of breast cancer cells and by extension, growth of breast tumors *in vivo*.

Reportable Outcomes:

- One manuscript published based on the work supported by this award (see appendix).

- Two manuscripts will be submitted this month based on work supported by this award.

- This award has supported the Ph.D. dissertation of one minority student Bobby Guillory who will defend his thesis by November of this year.

- Applied for another innovative idea (breast cancer) grant based on the work funded by this award (Pending).

Conclusion: From the in vivo studies, we can conclude that indeed for breast cancer, fetuin-A in the blood modulates breast carcinogenesis in a novel fashion. The studies suggest that fetuin-A sequesters TGF-beta such that it in not readily available to mediate its anti-carcinogenic properties in the developing mammary tissue. There is also a possibility that fetuin-A signals via the PI3 Kinase/Akt to promote tumor growth. The net result is that fetuin-a significantly shortens the tumor latency. We are on target to complete most of our proposed research in the next reporting cycle. We believe that we have obtained enough preliminary data that will enable us to apply for a regular ROI to continue this work. There will be a push for us to determine the motifs in both fetuin-A and TGF-beta that are necessary for the binding interaction. This will help us design drugs that could be used as chemoprevention against breast cancer. The support from DOD was absolutely crucial to get us to this stage and we are very appreciative of the opportunity. We hope to submit at least 3 more manuscripts based on the work supported by this grant.

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APPENDICES

EXPERIMENTAL CELL RESEARCH 315 (2009) 1875–1888

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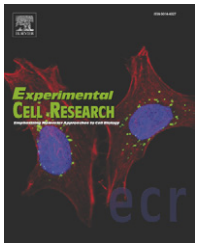

Research Article

Anchorage-independent growth of breast carcinoma cells is mediated by serum exosomes

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ABSTRACT

We hereby report studies that suggest a role for serum exosomes in the anchorage-independent growth (AIG) of tumor cells. In AIG assays, fetal bovine serum is one of the critical ingredients. We therefore purified exosomes from fetal bovine serum and examined their potential to promote growth of breast carcinoma cells in soft agar and Matrigel after reconstituting them into growth medium (EEM). In all the assays, viable colonies were formed only in the presence of exosomes. Some of the exosomal proteins we identified, have been documented by others and could be considered exosomal markers. Labeled purified exosomes were up-taken by the tumor cells, a process that could be competed out with excess unlabeled vesicles. Our data also suggested that once endocytosed by a cell, the exosomes could be recycled back to the conditioned medium from where they can be up-taken by other cells. We also demonstrated that low concentrations of exosomes activate MAP kinases, suggesting a mechanism by which they maintain the growth of the tumor cells in soft agar. Taken together, our data demonstrate that serum exosomes form a growth promoting platform for AIG of tumor cells and may open a new vista into cancer cell growth *in vivo*.

Introduction

The *in vitro* two-dimensional growth of cultured cells including tumor cells is well characterized, particularly the growth signals emanating from the integrin/extracellular matrix interaction [1]. For a number of years, ideas have bounced back and forth as to whether the growth signaling mechanisms in two-dimensional tissue culture can be extrapolated to the growth cues *in vivo* [2]. A number of *in vitro* culture systems have been developed including organotypic culture system, that takes into account the three-dimensional *in vivo* growth of tumor cells [3]. Suffice to say, the *in vivo* growth of tumor cells is complex because not only is it three-dimensional in character, there are numerous variables including stromal factors that affect growth [4]. Apart from the limitations imposed on it, the anchorage-independent growth of tumor cells in soft agar bears the closest resemblance to the *in vivo* growth when compared to the two-dimensional growth on plastic [5].

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Abbreviations: EEM, exosome enriched medium; EFM, exosome-free medium; HEMA, 2-hydroxyethylmethacrylate; AIG, anchorage-independent growth

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Serum, particularly fetal bovine serum is routinely used to supplement cell growth media. Serum has a number of adhesion or attachment factors which allow the cells to adhere and spread on the bottom of the culture flask to initiate growth signals. Apart from the well characterized adhesion proteins such as fibronectin, laminin, and collagen [6], serum also contains a myriad of factors whose role in cell growth is either defined or yet to be determined [7]. There are over 1000 proteins in serum, where some are at nanomolar or lower concentrations and others at micro molar or higher concentrations. In studies designed to measure the contribution of a particular growth factor, it is customary to use serum free medium supplemented with graded doses of the protein or factor. Such studies have been done to determine the growth factor most suited to support anchorage-independent growth [8]. Interestingly, in majority of such studies, in addition to the growth factor of interest, the medium was also supplemented with fetal bovine serum [9]. Serum proteins appear to be particularly important requirement for anchorage-independent growth. We have made an effort in the present study to identify the protein or group of proteins "platform" associated with anchorage-independent growth in serum.

We hypothesized that fetuin-A or a group of proteins which form a complex with or co-purify with it is/are responsible for anchorage-independent growth in serum. We had demonstrated that fetuin-A is one of the serum proteins that could support anchorage-independent growth in breast carcinoma cells [10]. Unfortunately fetuin-A easily associates with other serum proteins making it difficult to single it out as the principal driver of growth. Recent studies indicated that exosomes (nanovesicles) purified from HIV infected monocyte-derived macrophages and urine, contain fetuin-A among other serum proteins [11,12]. We hereby present data that demonstrate that serum proteins or factors (including fetuin-A) that confer anchorage-independent growth are associated with exosomes.

Materials and methods

Cells: A sub-clone of the breast cancer cell line BT-549 stably transfected with galectin-3 and re-named BT-549Gal-3, and MDA-MB-435, were kindly donated by Dr. Avraham Raz, Karmanos Cancer Research Institute. MCF-10A and MDA-MB-231, were purchased from ATCC. The cells were routinely cultured in DMEM/F12 supplemented with essential and non-essential amino acids, 100 μg/ml penicillin–streptomycin, 2.5 μg/ml Fungizone, 20 ng/ml epidermal growth factor, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 98 ng/ml cholera toxin, and 10% heat inactivated fetal bovine serum at 37 °C, 5% CO_2, in a humidified incubator. GFP-annexin-II expression plasmid was kindly donated to us by Dr Carl E. Creutz (University of Virginia). All the other reagents unless otherwise stated were purchased from Sigma (St Louis, MO).

Exosome purification protocol

To purify exosomes from fetal bovine serum, we used one batch of FBS from Gemini Bioproducts (West Sacramento, CA), one from PAA Laboratories, Inc. (New Bedford, MA), and two batches from Atlanta biologicals, Lawrenceville, GA. The serum (100 ml) was mixed with RPMI-1640 medium at a ratio of 50:50. This was centrifuged at 20,000 ×*g* to remove micro vesicles and other cellular debris and the resultant supernatant was centrifuged at 100,000 ×*g* for 10 h. The supernatant (exosome-free fraction) was carefully decanted and named exosome-free medium (EFM) while the resulting precipitate was dissolved in PBS and centrifuged at 200,000 ×*g* for 1 h. The resulting precipitate was dissolved in RPMI-1640 and constituted the exosome enriched medium (EEM). Both EEM and EFM were adjusted to a concentration of 6 mg/ml using serum free DMEM/F12 medium.

Anchorage-independent growth assays

The anchorage-independent growth assay (AIG) was modified for the 96-well culture plates. Aliquots of agar Noble (Difco) (0.8% w/v in water; 100 ml) were sterilized and stored at room temperature in conical flasks. The agar was melted, and kept in water bath at 50 °C. To make the bottom layer, one part of the melted 0.8% agar (700 μl) was mixed with 560 μl of 2× DMEM/F12 serum free medium containing either 140 μl of exosome enriched medium (EEM) or exosome-free medium (EFM). This final mixture (0.4% agar) was then added to wells at100 μl/well and allowed to gel at room temperature. To prepare the top layer (0.3% soft agar) 700 μl of 0.6% agar was mixed with 560 μl of 2× SFM containing the cells (500 cells/well) and either 140 μl of EEM or EFM. This mixture was then added to the bottom layer (100 μl/well) and allowed to gel at room temperature. After three weeks of growth the colonies were photographed. Alamar Blue (Biosource, Camarillo, CA) was also added to the wells to measure the growth potential of the colonies by monitoring the release of reducing metabolites as a read-out. In some experiments we used agarose (Invitrogen) instead of soft agar. We also assayed for 3-dimensional growth of tumor cells in Matrigel. Growth factor reduced Matrigel (BD Biosciences, Bedford, MA) from frozen stock was kept overnight at 4 °C to liquefy. This was then added to 96-well culture plate (100 μl/well) and allowed to solidify by incubating at 37 °C for at least 20 min. Serum free medium (SFM), EFM, and EEM were added (200 μl/well) on top of Matrigel followed by BT-549Gal3 cells (500 cells/well). The plates were then incubated in a humidified CO_2 incubator at 37 °C for two weeks.

Proteomic analysis of the proteins in exosomes

In-gel digestion

Equal amounts of proteins from EEM and EFM were loaded into SDS-PAGE (4–12% gradient gel), resolved and stained with Coomassie brilliant blue. Each lane was cut into 4 regions and each subjected to in-gel tryptic digestion [13]. Briefly, the gel regions were excised and washed with 100 mM ammonium bicarbonate for 15 min. The liquid was discarded and replaced with fresh 100 mM ammonium bicarbonate and the proteins reduced with 5 mM DTT for 20 min at 55 °C. After cooling to room temperature, iodoacetamide was added to 10 mM final concentration and placed in the dark for 20 min at room temperature. The solution was discarded and the gel pieces washed with 50% acetonitrile/50 mM ammonium bicarbonate for 20 min, followed by dehydration with 100% acetonitrile. The liquid was removed and the gel pieces were completely dried, re-swelled with 0.3 μg of modified trypsin (Promega) in 100 mM NH_4HCO_3, and digested overnight at 37 °C. Peptides were extracted by three changes of 60% acetonitrile/0.1% TFA, and extracts from each region were

combined separately, generating 4 samples per lane, and dried *in vacuo*. Samples were reconstituted in 30 μL 0.1% formic acid for LC–MS–MS analysis.

LC–MS–MS analysis and protein identification

Resulting peptides were analyzed using a Thermo Finnigan LTQ ion trap instrument equipped with a Thermo MicroAS autosampler and Thermo Surveyor HPLC pump, Nanospray source, and Xcalibur 2.0 SR2 instrument control. Peptides were separated on a packed capillary tip (Polymicro Technologies, 100 μm × 11 cm) with Jupiter C18 resin (5 μm, 300 Å, Phenomenex) using an in-line solid-phase extraction column (100 μm × 6 cm) packed with the same C18 resin [using a frit generated with liquid silicate] similar to that previously described [14]. The flow from the HPLC pump was split prior to the injection valve to achieve flow-rates of 700 nL–1000 μL min^{-1} at the column tip. Mobile phase A consisted of 0.1% formic acid and Mobile phase B consisted of 0.1% formic acid in acetonitrile. A 95 min gradient was performed with a 15 min washing period (100% A for the first 10 min followed by a gradient to 98% A at 15 min) to allow for solid-phase extraction and removal of any residual salts. Following the washing period, the gradient was increased to 25% B by 50 min, followed by an increase to 90% B by 65 min and held for 9 min before returning to the initial conditions. Tandem spectra were acquired using a data dependent scanning mode in which one full MS scan (m/z 400–2000) was followed by 9 MS–MS scans. Tandem spectra were searched against the bovine subset of the UniRef100 database using the SEQUEST algorithm. The database was concatenated with the reverse sequences of all proteins in the database to allow for the determination of false positive rates. Protein matches were preliminarily filtered using the following criteria: cross-correlation (X_{corr}) value of ≥1.0 for singly charged ions, ≥1.8 for doubly charged ions, and ≥2.5 for triply charged ions. A ranking of primary score (RSp) of ≤5 and a preliminary score (Sp) of ≥350 were also required for positive peptide identifications. Once filtered based on these scores, all proteins identified by less than two peptides were eliminated, resulting in false positive rates of <1%. The Sequest output was also filtered using IDPicker using a false positive ID threshold (default is 0.05 or 5% false positives) based on reverse sequence hits in the database. Protein reassembly from identified peptide sequences is done with the aid of a parsimony method recently described by Zhang et al. [15] which identifies indiscernible proteins (protein groups) that can account for the identified peptides.

Relative protein abundance (fold ratio changes) between exosome-free medium (EFM) and exosome enriched medium (EEM), were compared based on the spectral counts from two independent LC–MS/MS runs; corresponding to two aliquots from the same preparation run in separate lanes of SDS-PAGE gels (Tables 2). The use of sampling statistics has been shown to be a useful measure of relative protein abundance in label-free-LC–MS/MS shotgun proteomics. Recent studies have revealed a direct relationship between protein abundance and sampling statistics, such as percent sequence coverage, peptide count and spectral count. Most notably, the spectral count is the total number of tandem mass spectra (MS/MS) that are matched to the peptides of a protein. Liu et al. [16], demonstrated that spectral counts could be a reliable indicator of protein abundance with a linear range over 2 orders of magnitude by using a spectral count based measure; spectral sampling. In contrast to spectral counts, other sampling measures such as percent sequence coverage and number of peptides identified per protein did not have nearly as good of a linear correlation to actual protein abundance [16]. Moreover, spectral count has the highest technical reproducibility, followed by the less-reproducible peptide count and relatively non-reproducible sequence coverage [17]. We observed that the variance in spectral counts within samples was high due to the limited number of two technical replicates. This was especially true for lower abundance proteins having a low number of spectral counts; as even small differences in the spectral count number will have large effect on the variance. We note that our use of the ratio of spectral count change between exosome-free (EFM) and exosome enhanced media (EEM) is a qualitative and not a quantitative measure.

Transmission electron microscopy of the exosomes

The exosomal sample was fixed in 2.5% paraformaldehyde for at least 1 h, followed by 2 buffer washes (TBS) for 5 min each. The samples were then incubated with 50% ethanol for 15 min followed by 70% ethanol for 30 min. They were then mixed 1:1 with LR white resin in 100% ethanol for 30 min followed by 2:1 mix with LR white resin in 100% ethanol for 30 min. They were then

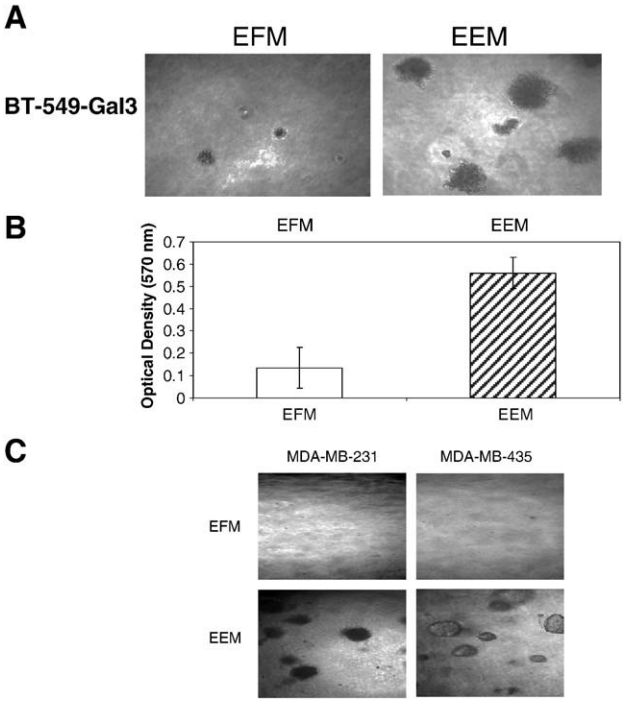

Fig. 1 – Bovine serum exosome mediated anchorage-independent growth of breast cancer cells in soft agar. Soft agar growth assays were set up in 96-well micro titer plates as described in Materials and methods. The cells were allowed to grow in soft agar either in the presence (EEM) or absence (EFM) of purified bovine exosomes. After 3 weeks the colonies were either photographed (panel A) or Alamar blue was added to the control wells and to the wells containing exosomes. After 4 h of color change, the plates were read at 570 nm (panel B). The growth promoting properties of the exosomes were also determined using other breast cancer cell lines (panel C).

Table 1 – Proteins found only in exosome enriched medium (EEM).

Accession number	Spectral counts		Description
	EFM	EEM	
UniRef100_UPI00005C0899	0	17	Similar to Filamin A
UniRef100_UPI00005C2264	0	8	Similar to myosin, heavy polypeptide 9, non-muscle, partial
UniRef100_P62935	0	6	Peptidyl–prolyl cis–trans isomerase A
UniRef100_Q2KJF4	0	6	Similar to Galectin-3 binding protein
UniRef100_Q76LV2	0	5	Heat shock protein HSP 90-alpha
UniRef100_Q27967	0	5	Secreted phosphoprotein 24 precursor
UniRef100_Q5E9F5	0	4	Transgelin-2
UniRef100_Q1RMK2	0	4	IGHM protein
UniRef100_P01267	0	4	Thyroglobulin precursor
UniRef100_UPI000051DE08	0	4	Tubulin alpha chain
UniRef100_O02717	0	4	Non-muscle myosin heavy chain
UniRef100_UPI00005BC732	0	4	Similar to fibulin 1
UniRef100_O18787	0	4	Elongation factor 1 alpha
UniRef100_UPI00005BFC16	0	3	Similar to Complement C4 precursor, partial
UniRef100_Q28107	0	3	Coagulation factor V precursor
UniRef100_P10096	0	3	Glyceraldehyde-3-phosphate dehydrogenase
UniRef100_UPI00005BC368	0	3	Similar to alpha 3 type VI collagen isoform 1 precursor
UniRef100_O46415	0	3	Ferritin light chain
UniRef100_UPI00005BC752	0	3	Similar to Plexin B2 precursor (MM1)
UniRef100_Q3SZZ9	0	3	FGG protein
UniRef100_UPI00005BEE04	0	3	Similar to stem cell growth factor precursor
UniRef100_UPI00005BC072	0	3	Similar to extracellular matrix protein 1
UniRef100_Q76LV1	0	2	Heat shock protein HSP 90-beta
UniRef100_Q32LP0	0	2	Unc-112-related protein 2
UniRef100_Q05443	0	2	Lumican precursor
UniRef100_UPI00005BDFA6	0	2	Similar to beta tubulin 1, class VI
UniRef100_UPI00005BE617	0	2	Similar to mannan-binding lectin serine protease 2
UniRef100_UPI00005BD7A2	0	2	Similar to talin 2
UniRef100_P28801	0	2	Glutathione S-transferase P
UniRef100_UPI00005C03A6	0	2	Similar to ubiquitin-activating enzyme E1 isoform 3
UniRef100_Q17QH6	0	2	Similar to collectin sub-family member 11 isoform a
UniRef100_UPI00005C030B	0	2	Similar to deleted in malignant brain tumors 1
UniRef100_P33672	0	2	Proteasome subunit beta type 3
UniRef100_Q3MHN2	0	2	Complement component C9 precursor
UniRef100_Q29451	0	2	Lysosomal alpha-mannosidase A peptide
UniRef100_UPI00005A1C47	0	2	Similar to karyopherin beta 1
UniRef100_P02676	0	1	Fibrinogen beta chain precursor
UniRef100_Q28106	0	1	Contactin-1 precursor
UniRef100_UPI00005C1679	0	1	Similar to Neogenin precursor, partial
UniRef100_UPI00005C18F6	0	1	Similar: C1q and tumor necrosis factor related protein 3
UniRef100_UPI00005C18F7	0	1	Similar to collagenous repeat-containing
UniRef100_Q3ZCJ2	0	1	Aldo-keto reductase family 1, member A1
UniRef100_Q2KJ63	0	1	Kallikrein B, plasma (Fletcher factor) 1
UniRef100_Q5E9B7	0	1	Chloride intracellular channel protein 1
UniRef100_Q3MHN0	0	1	Proteasome subunit beta type 6 precursor
UniRef100_O02808	0	1	Von Willebrand factor
UniRef100_UPI00005C164A	0	1	Similar to ADAM metallopeptidase
UniRef100_UPI00005C052B	0	1	Similar to Afamin precursor
UniRef100_UPI00005BB7FD	0	1	Similar to Carboxy-peptidase N subunit 2 precursor
UniRef100_Q3T149	0	1	Heat shock protein beta-1
UniRef100_UPI0000562815	0	1	Heat shock 27 kDa protein 1

Spectral counts are representative of two independent LC–MS/MS runs corresponding to two aliquots from the same preparation run in separate lanes of SDS-PAGE gels.

immersed in pure LR white resin for 60 min and then another fresh LR white resin for 60 min followed by LR white resin overnight in a rotator. They were then placed in gelatin capsules with fresh LR white resin and cured at 50 °C for 48 h. The sections were cut to 100 nm thickness and within 30 min, staining process was started. The sections were washed in TBS buffer 3 times for 30 s each then the grids blocked using 2% BSA for 30 min. The sections were then fixed in 2.5% glutaraldehyde for 30 s, washed in buffer and then 3 times in water for 30 s to remove salts and then stained for 30 s with 1% Uranyl acetate. The moisture was wicked away and then examined using a Phillips CM 12 TEM at 80 kV.

Up-take of purified serum exosomes by tumor cells

Exosomes purified as described above were labeled with rhodamine isothiocynate (Sigma) as described [18] and incubated with adherent BT-549Gal3 cells. Briefly, the total exosomal proteins were determined by the Bradford assay and 100 μg/assay were equilibrated in 100 mM carbonate buffer pH 9.0 by centrifugation at 10,000 ×*g* for 15 min in an ultra-filtration unit (MCO-100,000). The equilibrated exosomes were re-suspended in 100 μl of the same buffer and mixed with 100 μg/assay of rhodamine isothiocynate in DMSO. This was then incubated 2 h at room temperature and in the dark. After labeling, the exosomes were washed abundantly by centrifugation in the ultra-filtration unit to remove un-reacted rhodamine. Adherent breast tumor cells were grown on

microscope cover slips, starved for 48 h by growth in DMEM supplemented with 0.5% FBS, washed twice with Ca^{2+} and Mg^{2+}-free Dulbecco PBS (Invitrogen) and once with Ca^{2+} and Mg^{2+}-free HBSS (Invitrogen). Cells were then incubated for 30 min in HBSS containing 10 μg/ml rhodamine-labeled exosomes, 1 mM of divalent ions Ca^{2+}/Mg^{2+}, and the indicated concentrations of unlabeled exosomes. The cells were rinsed twice with cold PBS before and after fixation with −20 °C cold methanol for 10 min and the cover slips mounted on slides using Prolong with DAPI (Invitrogen). Up-take was visualized by fluorescent microscopy using a Nikon Eclipse TE-2000-E inverted microscope equipped with NIS-Elements AR software (Nikon, Melville, NY, USA).

Recycling of serum exosomes by tumor cells

BT-549Gal-3 cells were transfected with GFP-annexin A2 or mock transfected using Fugene6 according to the manufacturer (Roche) and serum-starved for 48 h. The mock transfected cells were incubated with rhodamine-labeled exosomes for 30 min at 37 °C, then washed twice with PBS at RT. The GFP-annexinA2 transfected and the rhodamine-labeled cells were respectively harvested by treatment with 2 mM EDTA and washed in cold PBS and once in HBSS supplemented with 1 mM Mg^{2+} and 1 mM Ca^{2+}. These were then mixed at a ratio of 1:3 (GFP-transfected cells to rhodamine-labeled cells), plated in six-well plates with cover slips and incubated for another 2 h at 37 °C. The cells were rinsed twice with cold PBS before and after fixation in −20 °C cold methanol for 10 min and the cover slips mounted on slides using Prolong with DAPI (Invitrogen). Up-take/recycling were visualized by fluorescent microscopy using a Nikon Eclipse TE-2000-E inverted microscope equipped with NIS-Elements AR software (Nikon, Melville, NY, USA).

MAP kinase signaling mediated by exosomes

Cells were plated in 10 cm dishes and serum-starved as above for 48 h. The adherent cells were then washed twice with PBS and once with 10 mM HEPES pH 7.2, 140 mM NaCl, 4.7 mM KCl (HBS) and incubated in HBS containing 1 mM Mg^{2+} and 1 mM Ca^{2+} without or with 0.1 mg/ml of exosomes for the indicated time points at 37 °C. The medium was rapidly discarded, replaced with ice-cold PBS and the dishes maintained on ice until the cells were harvested by scrapping. These were washed in the ice-cold PBS and the cell pellets stored at −80 °C until needed. In parallel experiments, cell culture dishes were pre-treated with poly(HEMA) as previously described [19]. The poly (HEMA)-treated dishes were sterilized by UV then washed twice with PBS. About 2×10^6 cells/dish that have been serum-starved as above were plated on the poly(HEMA)-treated dishes in HBS containing 1 mM Mg^{2+}, 1 mM Ca^{2+} without and with the indicated concentrations of exosomes (mg/ml) for 10 min or following a time course at 37 °C. MAP kinase activation was visualized by the detection of phosphorylated ERK1/2 by western blotting using antibodies to the dually phosphorylated ERK1 and ERK2. Total ERK as the loading control was detected using antibodies to either ERK2 or ERK1/2.

Results

Role of serum exosomes on anchorage dependent and independent growth of breast carcinoma cells

Our objective here was to determine the serum fraction (exosome-free or enriched) that supports anchorage-independent growth of breast carcinoma cells. The breast carcinoma cells were suspended in soft agar and allowed to grow either in the absence or presence of serum exosomes for at least 3 weeks. The data clearly show that AIG is robust only in those wells containing exosomes (Fig. 1). This serum fraction which we have named exosome enriched medium (EEM) in addition to common serum proteins such as albumin, also contains a unique set of proteins that are absent in exosome-free medium (EFM) (Table 1). The AIG assay was repeated at least 4 times with similar results. All the batches of fetal bovine serum we employed yielded exosomes that promoted AIG and the colonies grew in both soft agar and agarose at a similar rate. Not only did the cells growing in the presence of exosomes form large colonies with time, they were also healthy and actively released reducing metabolites into the conditioned medium, a hall mark of metabolic activity in growing cells (Fig. 1B). It can be argued that MDA-MB-435 is not an authentic breast cancer cell line [20]. However, its growth patterns in soft agar being similar to the other breast carcinoma cell lines, demonstrate that the ability of exosomes to influence anchorage-independent growth can be extrapolated to other tumor cells such as melanoma.

Tumor cells in serum free medium failed to grow in growth factor reduced Matrigel after two weeks of incubation (Fig. 2A). However, in EFM, the cells grew mainly as a monolayer on top of the Matrigel with extensive spreading. In EEM on the other hand, there were distinctive three-dimensional stellate colonies (Fig. 2C). These stellate colonies are typically observed when tumorigenic cells are grown in Matrigel [21]. Apart from anchorage-independent growth

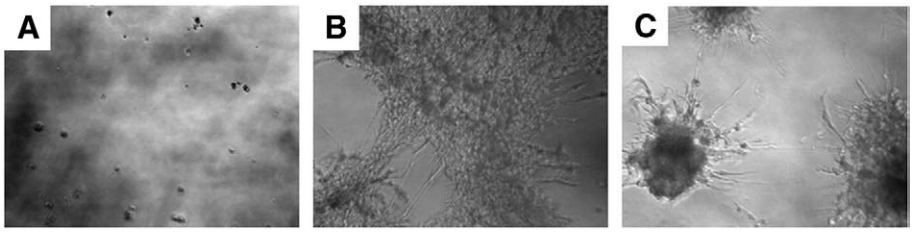

Fig. 2 – Bovine serum exosome mediated growth of stellate colonies of breast cancer cells in Matrigel. Liquid Matrigel (100 μl/well) was allowed to solidify at 37 °C in 96-well micro titer plate. The carcinoma cells were then added on the Matrigel (500 cells/well) in the presence of serum free medium (panel A), exosome-free medium (panel B) and exosome enriched medium (panel C). The cells were then incubated in a humidified CO_2 incubator for 14 days and then photographed.

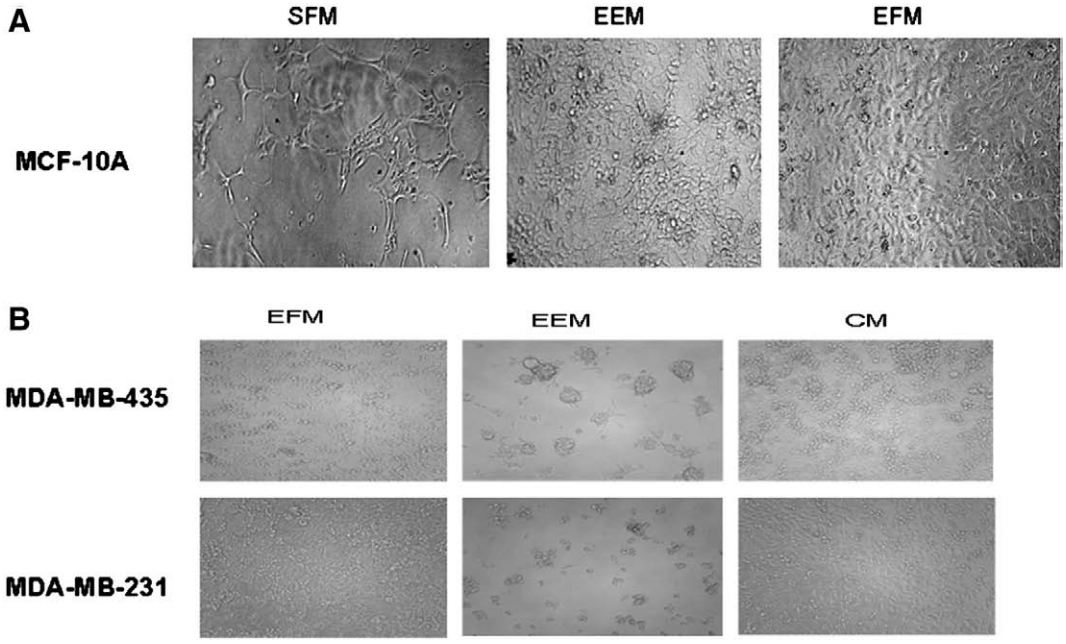

Fig. 3 – Bovine serum exosome mediated growth of normal and breast cancer cells on plastic. In panel A, MCF-10A were at 2×10^4 cells/well in 96-well micro titer plates in serum free medium, exosomal enriched medium (EEM) and exosomal free medium (EFM). The cells were allowed to grow for 5 days and then photographed. In panel B, one breast carcinoma cell line, MDA-MB-231 and a melanoma cell line, MDA-MB-435 were likewise plated at 2×10^4 cells/well in 96-well micro titer plates in EEM and EFM and in complete medium (CM) containing 10% fetal bovine serum.

in soft agar and Matrigel, we also examined the influence of exosomes on the growth of breast epithelial cells on plastic. In the presence of the vesicles, the tumor cells adhered and spread rapidly on plastic and with extended period of growth (seven days), the cells grew in tight aggregates on plastic (Fig. 3B). This type of growth is not uncommon in tumor cells [22], further re-enforcing the potential role of these vesicles on AIG. Due to the inability of normal breast epithelial cells (MCF-10A) to grow in soft agar, we speculated that they would also fail to grow on plastic in the presence of exosomes. On the contrary, the cells proliferated in the presence of the vesicles and as expected in vesicle free medium (EFM). However, in serum free medium, growth was minimal (Fig. 3A).

Analysis of proteins identified by mass-spectroscopy in both EEM and EFM

Transmission electron microscopy (TEM) revealed vesicles in EEM whose average diameter was approximately 150 nm (Fig. 4A). This average diameter was larger than that of bonafide cell derived exosomes with an average in diameter of between 60 and 100 nm

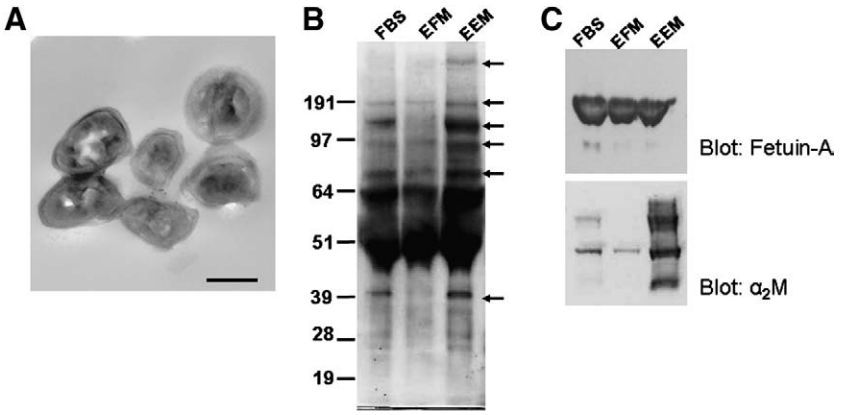

Fig. 4 – Proteins Associated with bovine serum exosomes. Panel A, transmission electron micrograph of exosomes purified from fetal bovine serum. The black line represents 100 nm. Panel B, aliquots of bovine serum, exosome-free serum, and exosomes were resolved in gradient SDS-PAGE and stained with coomassie brilliant blue. The protein bands were photographed and then all the proteins in each lane cut and analyzed by mass spec as described in Materials and methods. Panel C, proteins from bovine serum, exosome-free serum and exosomes were resolved in SDS-PAGE and then transferred to immobilon paper and probed for fetuin-A and alpha 2 macroglobulin.

Table 2 – List of relative fold change ratios of proteins in exosome enriched medium (EEM) versus exosome-free medium (EFM).

Accession number	Spectral counts		Ratio	Description
	EFM	EEM		
UniRef100_UPI00005BC629	5	95	19	Similar to Alpha-2-macroglobulin precursor
UniRef100_UPI00005BC5E5	1	15	15	Similar to pregnancy-zone protein
UniRef100_P07589	4	33	8.3	Fibronectin
UniRef100_UPI00005BB909	1	8	8	Similar to Ceruloplasmin precursor (Ferroxidase)
UniRef100_UPI00006830EC	5	35	7	Antithrombin III
UniRef100_Q3SZV7	1	7	7	Similar to hemopexin
UniRef100_P00735	5	32	6.4	Prothrombin precursor (Coagulation factor II)
UniRef100_UPI00005B628A	6	37	6.2	Hypothetical protein LOC540261
UniRef100_P97280	2	12	6	Inter-alpha-trypsin inhib. heavy chain H3 prec
UniRef100_Q2PQT6	1	6	6	Vinculin
UniRef100_UPI0000110A3C	5	27	5.4	Beta-actin
UniRef100_Q28194	3	16	5.3	Thrombospondin-1
UniRef100_Q3ZBS7	3	15	5	Vitronectin
UniRef100_UPI00005BC623	185	861	4.7	Similar: Alpha-2-macroglobulin precur. (A2-M)
UniRef100_UPI0000693DE0	66	298	4.5	Complement component 3
UniRef100_Q2UVX4	74	325	4.4	Complement C3 precursor
UniRef100_UPI00005C2942	2	8	4	Similar: Ig lambda chain V-I region BL2 precur.
UniRef100_Q32T06	1	4	4	Endopin 2C
UniRef100_Q0VCX1	1	4	4	Complement component 1, s subcomponent
UniRef100_UPI00005C201D	1	4	4	Similar: C4b-binding prot alpha chain precur
UniRef100_UPI00005C27D5	1	4	4	Similar: gamma-2a immunoglobulin heavy chain
UniRef100_Q06805	1	4	4	Tyrosine-protein kinase receptor Tie-1 precursor
UniRef100_Q95121	4	14	3.5	Pigment epithelium-derived factor precursor
UniRef100_Q03247	14	47	3.4	Apolipoprotein E precursor
UniRef100_UPI00005BC731	3	10	3.3	Similar to Fibulin-1 precursor isoform 1
UniRef100_P02584	3	10	3.3	Profilin-1
UniRef100_P17697	7	23	3.3	Clusterin precursor (Glycoprotein III)
UniRef100_P01966	22	72	3.3	Hemoglobin subunit alpha
UniRef100_Q28178	9	27	3	Thrombospondin-1 precursor
UniRef100_UPI00005BE9E4	2	6	3	Similar to Heparin cofactor II precursor (HC-II)
UniRef100_Q9N2I2	2	6	3	Plasma serine protease inhibitor precursor
UniRef100_P17690	1	3	3	Beta-2-glycoprotein 1 precursor
UniRef100_P02081	50	138	2.8	Hemoglobin fetal subunit beta
UniRef100_Q5E9B1	3	8	2.7	L-lactate dehydrogenase B chain
UniRef100_P02662	3	8	2.7	Alpha-S1-casein cursor
UniRef100_Q29RQ1	8	20	2.5	Complement component C7 precursor
UniRef100_UPI00005C18A3	2	5	2.5	Similar to Complement factor H precursor
UniRef100_UPI00005C1B6C	2	5	2.5	Perlecan
UniRef100_Q05B55	5	12	2.4	Hypothetical protein
UniRef100_UPI00005BFBFC	15	34	2.3	Similar to Complement C4 precursor
UniRef100_UPI00005C211E	13	28	2.2	Similar to Ig gamma-1 chain C region
UniRef100_Q0VCM5	15	31	2.1	Similar: Inter-alpha-trypsin inhib. heavy chain H1
UniRef100_P02070	31	62	2	Hemoglobin subunit beta
UniRef100_P22226	5	10	2	Cyclic dodecapeptide precursor
UniRef100_Q3SWW8	3	6	2	Similar to thrombospondin 4
UniRef100_Q2HJF0	3	6	2	Similar to Serotransferrin
rUniRef100_UPI00005C24B2	2	4	2	Similar to Laminin alpha-3 chain precursor
rUniRef100_UPI00005C16C9	1	2	2	Similar to CG9164-PA, isoform A
UniRef100_UPI00005BDE5A	1	2	2	Similar to Neuropilin-1 precursor
UniRef100_UPI00005BD502	1	2	2	Similar: Cofactor for Sp1 trans activation sub 3
UniRef100_Q5E956	1	2	2	Triosephosphate isomerase
UniRef100_UPI00005BFBFF	30	54	1.8	Similar to Complement C4 precursor, partial
UniRef100_Q3SZH5	10	18	1.8	Similar to Angiotensinogen
UniRef100_P06868	32	57	1.8	Plasminogen precursor (EC 3.4.21.7)
UniRef100_Q2KIF5	7	12	1.7	Vl1a protein
UniRef100_P02672	15	25	1.7	Fibrinogen alpha chain precursor
UniRef100_P01030	29	44	1.5	Complement C4 precursor
UniRef100_Q9BGI3	2	3	1.5	Peroxiredoxin-2
UniRef100_Q5EA67	57	84	1.5	Inter-alpha (Globulin) inhibitor H4
UniRef100_Q28085	22	32	1.5	Complement factor H precursor
UniRef100_Q3T052	58	84	1.5	Inter-alpha (Globulin) inhibitor H4
UniRef100_Q3SX14	24	33	1.4	Similar to Gelsolin

(continued on next page)

Table 2 ***(continued)***

Accession number	Spectral counts		Ratio	Description
	EFM	EEM		
UniRef100_Q0IIK2	289	390	1.4	Transferrin
UniRef100_Q9GLY6	18	24	1.3	Inter-alpha-trypsin inhibitor heavy chain2
UniRef100_Q3SZ57	25	33	1.3	Alpha-fetoprotein precursor
UniRef100_Q2KIU6	44	57	1.3	Similar to Complement factor B
UniRef100_P28800	14	18	1.3	Alpha-2-antiplasmin precursor
UniRef100_Q1RMN8	7	9	1.3	Similar to Ig lambda-like polypep. 1
UniRef100_P00978	27	34	1.3	AMBP protein precursor
UniRef100_Q3SZZ4	26	32	1.2	AMBP protein
UniRef100_UPI00005BDE33	82	99	1.2	Similar to inter-alpha globulin inhib. H2 polypep
UniRef100_Q2KIS7	5	6	1.2	Tetranectin precursor
UniRef100_P02453	5	6	1.2	Collagen alpha-1(I) chain precursor
UniRef100_Q3UZM0	23	27	1.2	8 days embryo whole body cDNA, RIKEN
UniRef100_P15497	30	35	1.2	Apolipoprotein A-I precursor
UniRef100_UPI00001116D7	13	15	1.2	Trypsin
UniRef100_Q0V8M9	62	69	1.1	Inter-alpha (Globulin) inhibitor H3
UniRef100_O46375	30	32	1.1	Transthyretin precursor
UniRef100_UPI00000718F2	39	41	1.1	Keratin 10
UniRef100_UPI00006830E5	17	17	1	Trypsin
UniRef100_Q58CQ9	4	4	1	Pantetheinase precursor
UniRef100_Q6LBN7	3	3	1	Lactoferrin
r-UniRef100_Q2KI19	2	2	1	Similar to CG10053-PA
UniRef100_Q2KIW5	1	1	1	Centromere protein U
UniRef100_UPI00005C2368	1	1	1	Similar to HGF activator preproprotein
UniRef100_UPI00005C11DB	1	1	1	Similar to Spectrin beta chain
UniRef100_UPI000016C268	1404	1389	1	Albumin
UniRef100_UPI00001AE6F7	55	51	0.9	Keratin 9
UniRef100_Q3MHN5	53	45	0.9	Vitamin D-binding protein precursor
UniRef100_Q2KJF1	18	15	0.8	Alpha-1-B glycoprotein
UniRef100_UPI0000E2470B	65	54	0.8	Cytokeratin-14
UniRef100_Q58D62	47	39	0.8	Fetuin-B precursor
UniRef100_P34955	158	130	0.8	Alpha-1-antiproteinase precursor
UniRef100_P18902	13	10	0.8	Plasma retinol-binding protein
UniRef100_UPI00001A389F	13	10	0.8	Plasma retinol-binding protein
UniRef100_Q2KIT0	4	3	0.8	Similar to collagen, type X, alpha 1
UniRef100_UPI0000D9CC8C	48	35	0.7	Similar to keratin 1 isoform 7
UniRef100_UPI0000167B80	62	45	0.7	Keratin 1
UniRef100_UPI0000110894	7	5	0.7	Beta-lactoglobulin
UniRef100_Q3ZEJ6	3	2	0.7	Serpina 3-3
UniRef100_Q4VAQ2	43	27	0.6	Keratin 2
UniRef100_UPI00005A1BEF	8	5	0.6	Similar to keratin 25D
UniRef100_P12763	400	235	0.6	Alpha-2-HS-glycoprotein precursor
UniRef100_Q3SZR3	12	7	0.6	Alpha-1 acid glycoprotein
UniRef100_UPI0000D9CC7B	15	8	0.5	Keratin 6A
UniRef100_P01044-2	22	11	0.5	Isoform LMW of P01044
UniRef100_UPI0000167F3F	22	11	0.5	Kininogen 1
UniRef100_Q9TTJ5	4	2	0.5	Regucalcin
UniRef100_P02465	4	2	0.5	Collagen alpha-2(I) chain precursor
UniRef100_UPI00006A12E2	4	2	0.5	Keratin 24
rUniRef100_UPI00005C1B5E	2	1	0.5	Similar to dynein, cytoplasmic, heavy polypep1
UniRef100_Q08E14	2	1	0.5	Similar to Collagen alpha 1(III) chain
UniRef100_P01035	2	1	0.5	Cystatin C precursor
UniRef100_UPI00006D113E	7	3	0.4	Keratin 5 isoform 20
UniRef100_Q2KJ62	23	9	0.4	KNG protein
UniRef100_UPI00005C202E	13	5	0.4	Similar to Apolipoprotein B-100 precursor
UniRef100_P08779	9	3	0.3	Keratin, type I cytoskeletal 16
UniRef100_UPI00005BDB2A	6	2	0.3	Similar to Apolipoprotein B-100 precursor
UniRef100_Q2KIQ9	6	2	0.3	Serpina3 protein
UniRef100_Q5DPW9	3	0	0	Cystatin E/M
UniRef100_P35445	2	0	0	Cartilage oligomeric matrix protein
UniRef100_P42916	2	0	0	Collectin-43 precursor
UniRef100_UPI00005C2037	2	0	0	Similar to Collagen alpha 1(V) chain precursor

Fold change ratio is calculated by the total number of spectral counts of a peptide/protein in the EEM divided by spectral count of EFM. Spectral counts are summation of two independent LC–MS/MS runs corresponding to two aliquots from the same preparation run in separate lanes of SDS-PAGE gels.

[23]. Serum vesicles (micro vesicles) precipitated at approximately 20,000 ×*g* lacked any biological activity such as support of anchorage-independent growth (data not shown). An examination of the distribution of two of the more abundant proteins in the EEM by sucrose gradient centrifugation revealed that while alpha 2-microglobulin was associated with the dense portion of the sucrose gradient, fetuin-A is associated with the less dense portion of the gradient (data not shown).

When the proteins in EEM and EFM were resolved on SDS-PAGE, it was evident that whereas most of the serum proteins appeared in both fractions, some were more concentrated in EEM (Fig. 4B, arrows). Among the proteins recovered in the EEM lane, a number of them were found only in exosomes by mass-spectroscopy (LC–MS/MS) (Table 1). The relative abundance of these proteins in either EFM or EEM was determined by use of spectral counts. The presence of at least two spectral counts was used as the threshold for peptide/protein identification. Given that the stringency during the LC–MS/MS was high and resulted in a measurable false positive rate of less than 1%, we conclude that a small spectral count is indicative of low abundance of the peptide, yet a high degree of confidence in the presence of that peptide in the sample. Interestingly, some of these proteins have also been reported in exosomes purified from other sources and are considered exosomal marker proteins. These include heat shock proteins HSP-90 [24], alpha tubulin [11], peptidyl prolyl trans isomerase [25,26], elongation factor 1 alpha [11], galectin-3 binding proteins [27], glyceraldehyde-3-phosphate dehydrogenase [11], and chloride intracellular channel protein 1[11]. By far the most abundant protein associated with serum exosomes is alpha 2-macroglobulin (Fig. 4C and Table 2). Interestingly other acute phase proteins synthesized in the liver such as fetuin-A and heat labile complement factors were also associated with the exosomes (Table 2). The concentration of fetuin-A in EFM was more or less comparable to its concentration in EEM (Table 2 and Fig. 4C). It is likely that the high enrichment of serum proteins in bovine serum exosomes may account for their rather large size compared to exosomes purified from cells.

Up-take of purified exosomes by tumor cells

Exosomes can be up-taken or endocytosed by cells via a mechanism that is not well characterized in the literature [28]. We show here that the purified labeled bovine exosomes can be endocytosed by breast carcinoma cells. After about 2 h of up-take, the exosomes appear to concentrate in the late endosomal compartment (Fig. 5A). This pattern of up-take is similar to that which has been observed by others [28]. Interestingly, when we incubated the cells with the labeled exosomes and excess unlabeled exosomes (Figs. 5B and C), the up-take of the former was blocked, suggesting that exosomes are endocytosed via a cell surface receptor/s.

Recycling of serum exosomes by tumor cells

It was interesting to observe that by giving the tumor cells just enough exosomes ~0.1 mg/ml, the cells were able to grow in soft agar for up to three weeks without the addition of more exosomes. This prompted us to question whether endocytosed exosomes can be recycled to maintain growth. To test this working hypothesis,

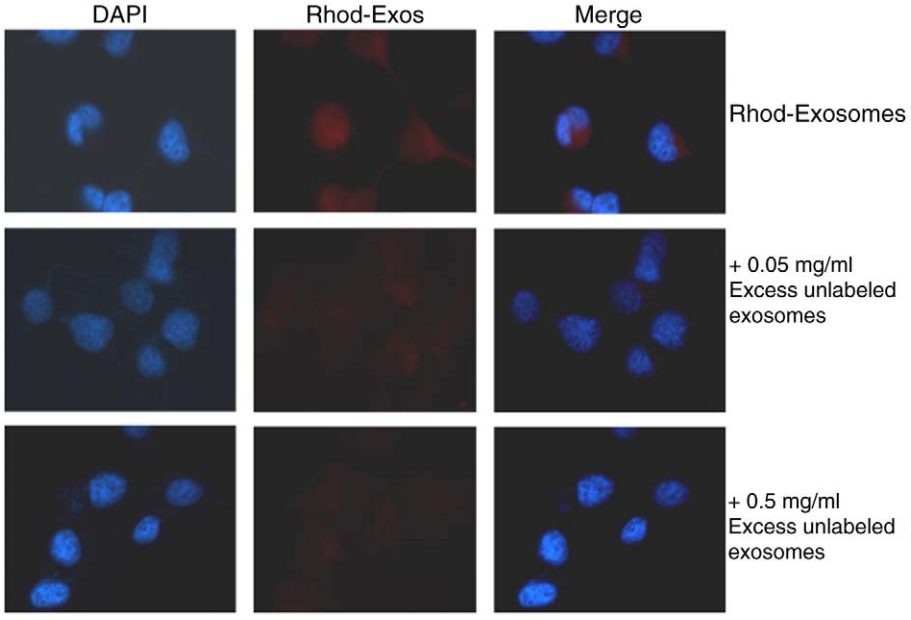

Fig. 5 – Up-take of purified bovine serum exosomes by tumor cells. Purified bovine exosomes were labeled with rhodamine isothiocynate, purified again by ultra-filtration to remove excess un-reacted rhodamine and then added to BT-549Gal-3 cells on cover slips which had been serum-starved for 48 h. The cells were incubated with labeled exosomes for 30 min in the absence and presence of excess unlabeled exosomes (0.05 mg/ml) and (0.5 mg/ml) respectively in HBSS containing divalent ions. The cells were rinsed twice with cold PBS and then fixed with −20 °C cold methanol for 10 min, cover-slipped and visualized by fluorescent microscopy. The experiment was repeated with MDA-MB-231 and MCF-10A cells with similar results.

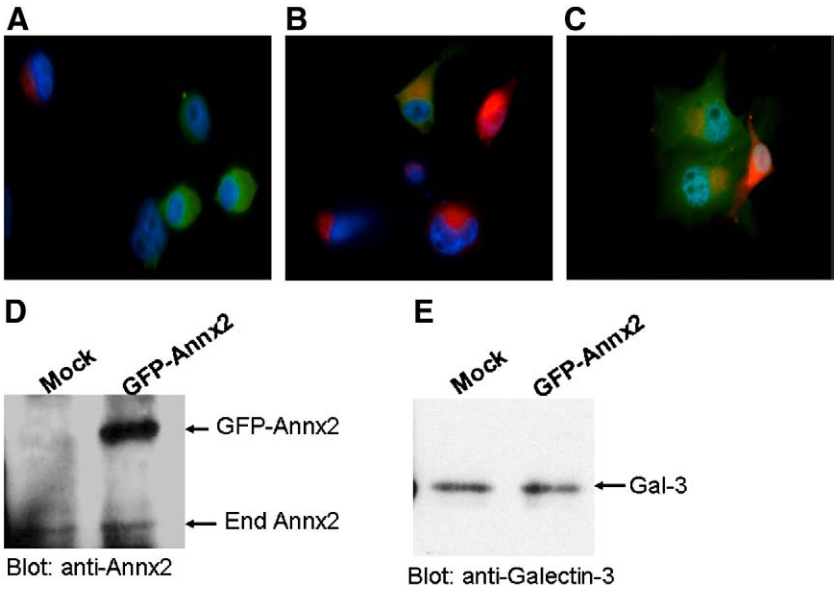

Fig. 6 – Recycling of bovine serum exosomes by tumor cells. BT-549Gal-3 cells were transfected with GFP-annexin-A2 or mock transfected using Fugene6 and serum-starved for 48 h. The mock transfected cells were then incubated with rhodamine-labeled exosomes for 30 min at 37 °C followed by washing with PBS at room temperature. The GFP-annexin-A2 transfected cells (green cells) and rhodamine-labeled (red) cells were harvested with 2 mM EDTA, washed in cold PBS and mixed at a ratio of 1:3 (green cells to red cells), plated in six-well plates on cover slips and incubated for 10 min (Panel A) and then for 2 h at 37 °C (Panels B and C). The GFP-annexin-A2 and mock transfected cells were also detached with EDTA and incubated separately in HBSS in siliconized tubes for 2 h at 37 °C. Exosomes were isolated and purified from the conditioned medium as described in Materials and methods. The cellular exosomes were resolved in SDS-PAGE, transferred to immobilon membranes and probed with annexin-2 (Panel D) and galectin-3 (Panel E) antibodies.

we transfected BT-549-Gal3 cells with GFP-annexinA2 and incubated the cells with rhodamine isothiocynate labeled exosomes. For the first 30 min. we could see only red and green cells (Fig. 6A). However, after 3 h of incubation, we mostly observed red and orange cells (Figs. 6B and C). This means that the cells that had endocytosed exosomes, released some back to the conditioned medium where they were up-taken by GFP-annnexinA2-transfected cells (Figs. 6B and C). In Fig. 6C, it is evident that the two GFP-annexinA2 transfected cells had endocytosed the released exosomes that were now concentrated in the late endosomal compartment, where they co-localized with annexinA2 which is also secreted from the cells via the recycling pathway of exosomes. We further demonstrated this by isolating and purifying exosomes from GFP-annexinA2 transfected cells and showing that GFP-annexinA2 is indeed recovered in the exosomal fraction (Fig. 6D). Members of annexin family of membrane proteins are routinely observed in cellular exosomes and can be considered markers of cellular exosomes [29]. In addition, galectin-3, another marker of cellular exosomes [29], was shown to be present in the exosomes purified from the tumor cells (Fig. 6E).

Map kinase signaling mediated by exosomes

We next investigated the role of exosomes in transmitting growth signals to cells on plastic or non-adhered cells growing on poly-(HEMA) coated dishes. It was interesting to note that on plastic and in the presence of Ca^{2+}/Mg^{2+} ions, ERK was activated (phospho-ERK) within 10 min of exosome addition and the activity diminished after 90 min (Fig. 7A). This is the pattern of activation that is usually observed when one plots a time course of ERK activation [30]. However in the presence of a low concentration of exosomes (0.1 mg/ml), ERK was again activated within 5 min but the signal remained strong for at least 240 min (Fig. 7A). This mode of activation is in line with adhesion and spreading of the cells on plastic in the presence of exosomes. In non-adherent cells, ERK activation was observed in the presence of low concentrations of exosomes (0.05 mg/ml) but at elevated concentrations approaching 0.5 mg/ml, activation of ERK was inhibited (Fig. 7B). This has been a consistent observation in at least 3 different experiments. Again as observed in adhered cells, in the presence of low concentrations of exosomes (0.1 mg/ml), the phosphorylated signal in ERK1/2 remained strong for at least 2 h (Fig. 7C). Surprisingly the ERK1/2 activation signal in anchorage-independent cells in the presence of exosomes (0.1 mg/ml) was evident even after 18 h of growth (data not shown). A similar sustained activation of ERK in non-adhered cells was recently reported in ovarian cancer cells [31].

Discussion

In the present studies, we have re-visited a long standing question regarding the identity of factor(s) in fetal bovine serum that mediates anchorage-independent growth of tumor cells. A number of growth factors such as IGF, EGF, TGF-β, βFGF and PDGF have individually and as a group, been shown to play a role in

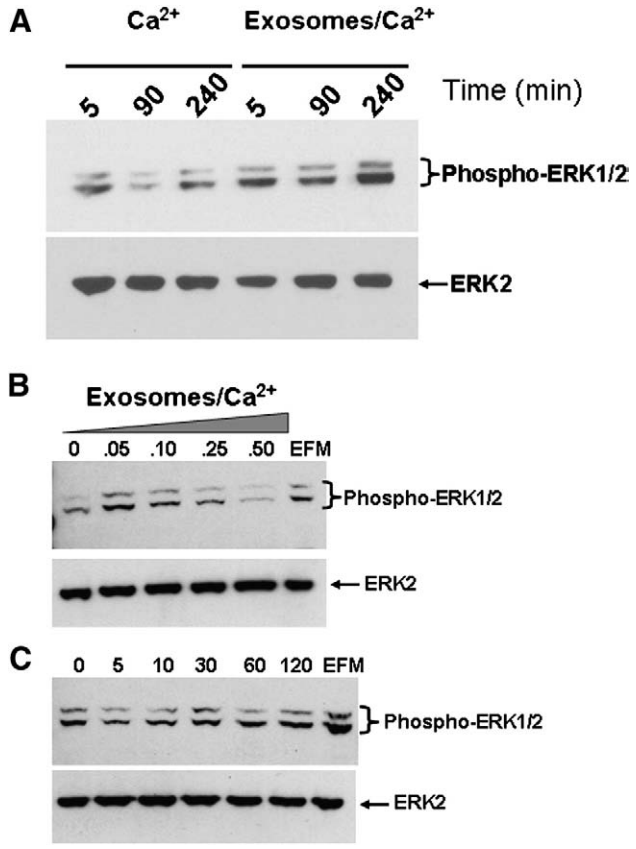

Fig. 7 – MAP Kinase signaling mediated by bovine serum exosomes. BT-549Gal3 cells were plated in 10 cm. dishes and serum-starved for 48 h. The adherent cells were incubated in HBS containing 1 mM of the divalent ions (Mg^{2+}/Ca^{2+}) for the indicated time points (Panel A). The cells were scrapped, washed in ice-cold PBS and stored at −80 °C until needed. In parallel experiments, cells (2×10^6 cells/dish) were plated on poly(HEMA)-treated dishes in HBS containing the divalent ions (Ca^{2+}/Mg^{2+}) without or with indicated concentrations of exosomes (mg/ml) for 10 min (Panel B). EFM (0.5 mg/ml) was also included as a positive control. In Panel C, the cells were incubated with 0.1 mg/ml of exosomes on poly(HEMA) coated wells for the indicated time points. The EFM (0.1 mg/ml) was also incubated with the cells for 120 min as a control. MAP kinase activation was visualized by detection of phosphorylated ERK1/2 by western blotting using antibodies to the dually phosphorylated ERK1 and ERK2. Total ERK (loading control) was detected using antibodies to ERK2.

anchorage-independent growth of tumor cells [8,32,33]. However, even after all these growth factors were included as a cocktail in serum free medium and mixed with soft agar, colony formation by tumor cells was still less compared to growth in the medium containing serum [34]. It is in this background of looking for serum proteins that may influence anchorage-independent growth that we questioned whether fetuin-A (a serum glycoprotein) plays a role in anchorage-independent growth of tumor cells. In our earlier studies we determined that antibodies to this glycoprotein reduced anchorage-independent growth potential in soft agar (data not shown). However, fetuin-A in addition to being secreted into the blood as a free protein, is also bound to other proteins [35] and has been shown to be associated with exosomes in the serum [11,12].

In the present analyses, our goal was to identify the serum fraction (exosome-free or exosome enriched) that supports anchorage-independent growth (AIG) of breast carcinoma cells. The data clearly show that the membranous fraction of fetal bovine serum (contains exosomes) is the one that mediates AIG of the tumor cells. The *in vitro* growth of tumor cells in soft agar is regarded as an excellent predictor of tumor growth *in vivo* [5]. The ability of exosomes to mediate the growth of stellate colonies [21] in Matrigel further underscores the potential role of exosomes in the *in vivo* tumor growth. Whereas Matrigel also supported the growth of tumor cells in EFM, the cells mainly grew in 2-dimensions and not inwards or upwards in this medium.

Therefore in order for us to dissect *in vivo* tumor growth mechanisms, the interaction between these exosomes and carcinoma cells deserves a rigorous analysis. The exosomes are able to promote the growth of both normal (MCF-10A) and breast carcinoma cells. Growth signals for anchorage-independent growth of tumor cells are well documented in the literature [2]. In normal cells (such as MCF-10A) that are attached or adhered to the substratum, the engagement of integrins by extracellular matrix proteins transmits growth signals that can also crosstalk with those emanating from growth factor/receptor interaction again resulting in cell growth [2]. However, in normal cells, lack of adhesion to the substratum results in anoikis or programmed cell death in case of epithelial and endothelial cells [2]. Transformed cells on the other hand are capable of transmitting growth signals whether or not they are attached to the substratum. However, the precise mechanism by which transformed cells coordinate the cross talks among the various growth signals while simultaneously inhibiting the apoptotic signals is poorly understood.

Despite the anchorage independence of transformed cells, they still require a number of growth factors, some of which the cells synthesize (autocrine) and some obtained from the serum. Our data suggest that in addition to providing the breast carcinoma cells with the growth factors such as TGF, the membranous portion of serum (contains exosomes) provide unique growth platforms. The vesicles could in theory concentrate growth factors on the surfaces of tumor cells. They could also promote cell to cell aggregation resulting in the enhancement of growth signals. The bovine vesicles just like the human exosomes, could also be derived from a variety of cell types including epithelial cells [36], macrophages [37], and platelets [38]. Some of the proteins could be imbedded in the vesicles while others such as fetuin-A could be on the surface. A number of serum proteins associated with exosomes have been implicated in anchorage-independent growth of tumor cells in soft agar. Studies by Calhoun et al. [39], have demonstrated that knockdown of cyclophilin A expression using RNAi in U2OS cells resulted in disruption of the F-actin structure and decreased anchorage-independent growth, proliferation and migration. Interestingly, Emmprin (CD 147), a cell surface glycoprotein that plays a critical role in cellular transformation and anchorage-independent growth, is a receptor for cyclophilin A [40]. The other protein that is abundantly expressed in the exosomes, HSP-90 alpha also plays a role in anchorage-independent growth together with the other members of the family. The majority of known substrates for the 90 kDa heat shock protein (HSP-90) are signal intermediates of clinical importance such as kinases [41,42]. For example, activated Cd42-associated kinase (AcK1) expression in

LNCaP cells enhanced anchorage-independent growth *in vitro* and accelerated tumorigenicity in nude mice. However, its activity required the binding of HSP-90 beta [43]. Furthermore, the expression of the small heat shock protein (HSP-27) is associated with increased anchorage-independent growth, increased invasiveness and resistance to chemotherapeutic drugs, poor prognosis and reduced disease free survival [44]. Studies by Anand et al. [45] have demonstrated that the gene encoding protein elongation factor (eEF-1 alpha) is amplified in 25% of primary ovarian tumors and is highly expressed in approximately 30% of ovarian tumors. They further showed that eEF-1alpha enhances focus formation, enhances anchorage-independent growth and decreases doubling time of rodent fibroblasts.

Apart from the above mentioned serum proteins that are exclusively associated with exosomes, others such as alpha2-macroglobulin are concentrated in them but also occur in the exosome-free fraction of serum. Another group of proteins which include fetuin-A, is more or less equally distributed in both EEM and EFM. Fetuin-A may after all, act as an opsonin to facilitate the up-take of the exosomes by the tumor cells [46]. Some of the exosomal associated serum proteins, particularly those that are loosely attached to their surfaces, may have specific receptors on the tumor cells through which they transmit their growth signals. However, the transit time of the proteins on the cell surface may be limited because by 10 min of adding exosomes to the cells, they are endocytosed. Once inside the cells, the exosomal proteins could directly promote anchorage-independent growth and then channeled to the degradative pathway [47]. They could also be diverted into the recycling pathway of exosomes [48]. There are bovine serum proteins including fetuin-A that are routinely observed in exosomes purified from cells [11]. The assumption therefore is that these are bovine serum exosomal proteins that are part of the recycling exosomal pathway.

We have directly demonstrated that labeled bovine exosomes taken up by one breast tumor cell can eventually exit this cell and be up-taken by another. As the serum exosomes including their assortment of proteins are up-taken by cells on hand, the released exosomes on the other hand bring out with them a unique set of cellular proteins. For example, we have demonstrated that galectin-3, annexin-2GFP (in transfected cells) and endogenous annexin-2 are part of the repertoire of proteins secreted in exosomes from tumor cells. Both of these have been observed in cellular exosomes [29]. Galectin-3 is one of the cellular proteins that have been implicated in anchorage-independent growth of tumor cells in soft agar [49]. It is thus tempting to speculate that exosomal galectin-3 is one of the key proteins with anchorage-independent growth promoting properties. The up-take of serum exosomes by tumor cells could in theory speed up the release of the cellular exosomes (bearing these unique proteins) which in turn are up-taken by neighboring cells to influence anchorage-independent growth.

In order to begin to understand mechanistic pathways by which exosomes influence anchorage-independent growth in tumor cells, we analyzed MAP kinase signaling in the interaction between tumor cells and purified exosomes. This is one of the key signaling pathway in tumor cells and in particular breast carcinomas that is responsible for maintenance of AIG [50,51]. Obviously there are cross talks between MAP kinase pathways and other pathways that have also been heavily implicated in anchorage-independent growth such as PI3 kinase/Akt [52]. The strong activation of ERK1/2 in the tumor cells by the bovine exosomes in the absence of growth factors such as EGF, suggest that these vesicles act in a synergistic manner with other growth factors to promote AIG. The ability of low concentrations of exosomes to maintain anchorage-independent growth in the tumor cells argues for recycling of the exosomal derived growth factors and is consistent with sustained activation of ERK1/2.

In summary, we have demonstrated that serum factors necessary for anchorage-independent growth of tumor cells in soft agar are concentrated in the exosomal compartment of serum and that these exosomes are most likely recycled to maintain growth. It is highly likely that human serum exosomes will also display a similar protein profile as the bovine vesicles, and with the potential to drive the *in vivo* growth of tumors particularly of the breast. Work is on going in our laboratory to identify cell surface receptor/s responsible for the endocytic up-take of the bovine exosomes. The identity of the key proteins that drive anchorage-independent growth, whether originating from the exosomal compartment of tumor cells or bovine serum, is another focus area.

Acknowledgments

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School of Medicine
Department of Biochemistry & Cancer Biology

September 3, 2009

Ms. Juanita Livingston
Technical Editor
Information Management
Fort Detrick, MD

REF: W81XWH-07-1-0254 (Revised Progress Report)

Dear Ms. Livingston:

Please find enclosed my revised progress report for the above referenced Award. I have responded to the reviewer comments as follows:

Query # 1: The PI also stated that current results indicate that fetuin-A is no a key driver of the PI3 kinase/Akt signaling…….

Response: We did not show the PI3Kinase/Akt data in our initial report. We have now included these in our revised version.

Query # 2 (Format/editorial issues). This report is not in compliance with some of the tasks outlined in the Statement of Work (SOW).

Response: We agree with the reviewer. Because we had to look at alternative explanation for our findings, we deviated slightly from the original SOW. We therefore modified our SOW and asked for approval. I was given a verbal approval and so we are now following the modified SOW which is included in the appendices.

Query # 2b: This annual report does not list key research accomplishments in a separate section.

Response: We apologize for this. We have now included key accomplishments in a separate page.

Query # 3. The PI is not in compliance with original SOW that was submitted. For Task 2, the PI introduced….

Response: We have included the PI3 kinase/data that was missing and made a case as to why we decided to also look at the TGF-beta pathway. We apologize for introducing a modified animal model i.e. transplanting the tumors. We modified our animal protocols to do these studies because we wanted to understand the mechanisms by which fetuin-A shortens the tumor latency. We have now stopped these experiments and the preliminary date we obtained will not be reported because we did not follow the rules. Also we have not done the RNA and DNA microarray studies. We hope to use another Award mechanism to do those studies because of the expense involved and so they are not included in the report.

Sincerely,

Josiah Ochieng, Ph.D.
Professor

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OCHIENG, JOSIAH (Modified statement of work)

Statement of Work
Role of Fetuin-A in Breast Tumor Cell Growth.

Task 1 To elucidate the role of fetuin-A in mammary tumorigenesis as assessed by fetuin-A knockout and polyoma virus middle T antigen transgenic mice (Months 1-18).

a) Start a breeding protocol by crossing $Fet^{-/-}$C57/BL-6 mice with $Fet^{+/+}PymT^{+}$ in FVB/N background (Months 1-5)

b) $Fet^{-/-}PymT^{+}$ and $Fet^{+/+}PymT^{+}$ mice will be moved from the breeding protocols to experimental groups (Months 5-10).

c) Repeat task # 1b, to confirm data (Months 10-15).

d) Analyze data (Months 15-29)

- Immunohistochemistry to show the expression of phosphorylated Akt , Phosphosmad2/3 in the tumors and other growth related signaling molecules (Months 15-24).
- To determine the influence of fetuin-A on the metastatic spread of breast tumors to the lungs (Months 24-29)

Task 1 has been completed and manuscript is preparation.

Task 2 To define the contribution of fetuin-A in the *in vitro* growth and signaling of human breast carcinoma cells (Months 18-36)

a) Purify fetuin-A from fetal bovine serum using a modified protocol. (Months 18-24)

b) Use the fetuin-A purified from fetal bovine to assay for growth and cell signaling in human breast carcinoma cells (Months 24-30)

c) Use the modified protocol to purify fetuin-A from human serum purchased from Sigma (Months 30-32)

d) Use the purified human fetuin-A (alpha 2HS glycoprotein) to assay for growth signaling mechanism in human breast epithelial cells (Months 32-36).

Justification for changes in the statement of Work.

In the original statement of work, it was anticipated that **task one** would be completed in 18 months. This was not possible because it took us longer than expected get the breeding protocol working and move the animals into the experimental protocols. We have just completed the last analysis and are now preparing the manuscript for submission. In the original SOW, we only stated that analysis would be done in months 15-29 but did not indicate the kinds of analysis to be done. I have now indicated all the analysis we have done on the tumors that were obtained from both the fetuin-A null and wild-type mice.

In Task 2, we proposed to use the original protocol of one step affinity chromatography (using WGA) to purify fetuin-A from human serum. We quickly noticed that using this purification protocol, we were still getting alpha 2 macroglobulin as a major contaminant of fetuin-A purified from both fetal bovine and human sera. Alpha 2-macroglobuling has also been shown to have the capacity to activate PI3 Kinase/Akt. We therefore had to come up with a new protocol. We developed a protocol which for the first time has been able to separate alpha 2 macroglobulin from fetuin-A. As expected most of the cell attachment and growth activities are associated with fetuin-A and not α-2-macroglobulin. Now that we have established this method, we hope to begin to purify large quantities of human fetuin-A using this method (Months 30-32). This is the first time we have been able to see a single band of fetuin-A in both SDS-PAGE-coomassie and western blot.